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TRANSLOCATED HEAT INJURY[1]

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In the course of my studies of heat activation of virus infections (8), I occasionally observed that an injury somewhat resembling that due to direct heat occurred on unheated leaves of the same plant. When I found that this injury was not due to a direct effect of heat and occurred principally on leaves opposite heated leaves, I undertook further studies to determine the condition for expression of this translocated injury.

METHODS

Translocated heat injury is defined as the injury to unheated leaves which results when other leaves of the same plant are heated. This study, except as noted otherwise, refers to the injury which resulted on one of the primary unifoliate leaves of Pinto bean (*Phaseolus vulgaris* L.) or Blackeye cowpea [*Vigna sinensis* (Torner) Savi] when the opposite leaf was heated. Direct injury is that which resulted on the heated leaf.

Beans and cowpeas growing for about 15 days in 4-inch pots of sterilized soil in the greenhouse at about 20 C were principally used. National Pickling cucumbers (*Cucumis sativus* L.), seedling peaches [*Prunus persica* (L.) Batsch] and seedling pigweed (*Chenopodium amaranticolor* Coste & Reyn.) were used to a lesser extent. Abrasion with Celite was used as in mechanical inoculation with viruses, except that water usually was used instead of a virus suspension. Heating was by immersion of attached leaves in a water bath. Injury was rated on a decimal scale of 0 to 10; 1 indicated no injury, 5 that the leaf was half dead, and 10 that the leaf was dead. This rating method I have shown (8) to be well correlated with injury as determined by green weight. Temperatures are given as °C. Virus inoculation was by standard methods.

The pathogens studied were tobacco mosaic virus (TMV), peach yellow bud mosaic virus (YBV), and bean rust [*Uromyces phaseoli* (Pers) Wint.].

RESULTS

MANIFESTATION OF TRANSLOCATED INJURY. Translocated injury was never apparent unless the heated leaf was severely injured, and usually not unless the heated leaf was killed. Direct heat injury was first manifested as a reduction in turgidity which was fairly uniform over the entire leaf; it could be evident within 3 seconds while the leaves were still in the water at temperatures from 75 to 90°; it could be delayed as much as 15 minutes after removal from the water (as with treatments of 8 sec at 60°), or could be delayed as much as 4 days (as in some treatments of 8 sec at 55°.) Translocated injury, first evident as a diffuse loss of turgidity and change of color, never appeared sooner than 0.5 hours, and in ordinary trials never appeared later than 4 hours after heating and abrading. Recovery from translocated injury was manifest as bands of turgid tissue along the veins in formerly flaccid tissue; these bands of turgid tissue increased in width as recovery progressed. With extensive translocated injury, recovery was rarely complete; the injury remained as intervenal necrosis (fig 1), which could involve up to 90 % of the leaf area (injury rating of 9). Partial recovery usually occurred without any special treatment; complete recovery was common. Recovery was favored by detachment of the heated leaf; if the heated leaf was detached within 3 hours of heating, recovery was usually complete. Ratings of heat injury usually were made at about 24 hours after heating, after which time no further recovery occurred.

ABRASION. Abrasion of leaves increased direct injury only slightly. For example, in one trial, the ED50 was about 24 seconds at 55° for unabraded leaves and 18 seconds for abraded leaves. For translocated heat injury, abrasion of the untreated bean and cowpea leaves was almost essential. Translocated heat injury to unabraded cowpea leaves was observed only three times and this injury was slight at dosages of 8 and 16 seconds at 90°. This dosage was much greater than necessary to produce translocated injury with abrasion, and even this effect could not be repeated in two trials. While other types of injury (such as abrasion with dry carborundum, brushing

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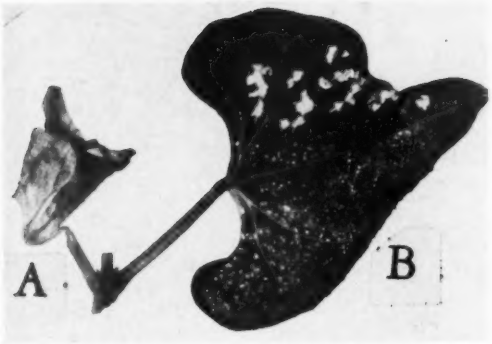

FIG. 1. Translocated heat injury in bean. The right (*lower*) half of the right leaf (B) was inoculated with rust January 30. This entire leaf was abraded with Celite at 6 AM, February 4. The left leaf (A) was heated 30 seconds at 65 C at 6 AM, February 4. Photographed February 8. The heated leaf was killed, and the healthy half of the non-heated leaf was severely injured. Rust infection reduced translocated heat injury.

with a wet or dry brush without carborundum, rubbing leaves with the fingers, scratching the leaves with needles, and pressing the leaves with the fingers) increased translocated injury, these types of injury were less effective than abrasion with wet Celite.

While abrasion of the heated leaf increased direct heat injury to bean and cowpea and was almost essential on the unheated leaf to bring about translocated heat injury, abrasion of the heated leaf consistently decreased translocated injury. In three trials with 24 pairs of leaves (heat dosages ranging from 1–16 seconds at 65°) the average translocated injury rating of cowpea was 0 when there was no abrasion of heated or unheated leaf; 0 when only the heated leaf was abraded; 3 to 8 when only the unheated leaf was abraded, and 1 to 2 when both leaves were abraded. This reduction in translocated injury by abrading the heated leaf has not been explained. Plausibly, the reduction might be explained by a heat-induced toxin released or inactivated by the abrasions, but since no toxin has been demonstrated, this explanation has little support. With peach and pigweed, severe translocated heat injury occurred on some unabraded lower leaves when the upper leaves of the same plants were heated for 5 to 20 seconds at 65°. Abrasion seemed to have little effect on translocated heat injury on these plants.

On bean and cowpea, translocated injury usually occurred only at the site of abrasion. If only the distal half of the leaf was abraded, translocated injury was manifest only in the distal half. Similarly for the proximal half or the center or edge of the leaf. Abrasion of the upper leaf surface was more effective in inducing translocated injury than was abrasion of the lower surface and complete recovery was more likely if the lower surface was abraded.

PHOSPHATE. Beans and cowpeas are commonly inoculated with viruses by rubbing the leaves with an aqueous mixture of virus, abrasive, and 1 % K_2HPO_4 (phosphate). Occasionally this added phosphate causes direct injury in routine inoculations, and this injury increased with an increase in temperature. Surprisingly, however, translocated heat injury was reduced by phosphate. In two trials using bean and cowpea and using 4 to 16 seconds at 65° to produce translocated heat injury, the average rating for translocated heat injury was 5.7 for nine plants of which the unheated leaves were abraded with Celite suspensions in water and 2.3 for nine plants abraded with Celite suspensions in phosphate. In four trials the average translocated heat injury was 3.3 for 14 plants of which one leaf was heated in water, and 1.6 for 14 plants of which one leaf was heated in phosphate.

HEAT DOSAGE. With increasing duration of heating, direct injury to the heated leaves increased with an approximately straight line relationship on arithmetic log scales. This response showed an ED50 (dosage for 50 % injury) of about 500 seconds at 45°, 75 seconds at 50°, 16 seconds at 55°, 3 seconds at 60°, and 1 second at 65° (fig 2) or the temperature coefficient was about 25 for this range. Complete killing by translocated injury was never attained and the ED50 for translocated injury is taken as a rating of 4 which is about half of the maximum translocated injury in any trial. The ED50 for translocated injury was about 140 seconds at 55°, 30 seconds at 60°, 10 seconds at 65°, and 3 seconds at 70°, or the temperature coefficient was about 14. Data for translocated heat injury were much more erratic than for

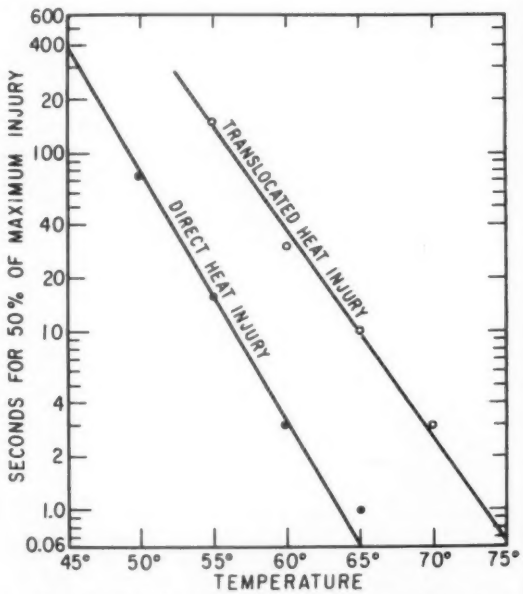

FIG. 2. Relationship of heat dosage and temperature for direct and translocated heat injury to bean and cowpea.

direct injury, and the values and temperature coefficients for translocated injury are less reliable than for direct injury. It required about ten times the heat dosage to produce translocated injury as to produce direct injury.

AGE OF PLANTS. For studies of the effect of age of plants on translocated heat injury, only trials where plants of a series of ages were treated in the same trial and where heatings were made between 6 AM and 8 AM are included. Age of plants is given as days from seeding, and therefore the treated leaves were much younger than the ages given. Plants were started at about 18°, which is much below the optimum for beans, and primary leaves were about three centimeters long at 10 days and about eight centimeters at 23 days. Treatments included 1 to 30 seconds at 60° and 1 to 30 seconds at 70°, and most of these treatments killed the heated leaf. Within each trial, plants of different ages were given the same heat treatments. The average rating for translocated injury was 6 for five 23-day bean plants, 3.8 for fourteen 19-day plants, 3.3 for five 18-day plants, 0.7 for six 16-day plants, zero for four 13-day plants, and zero for eight 12-day plants. In some trials not in the age of plants series slight injury occurred on plants 11 days of age but this was exceptional. With cowpea, translocated injury could be produced on younger plants than with bean.

HUMIDITY. In most trials the potted plants were placed on the greenhouse benches in relatively dry air after heating. Since collapse and drying out of tissues was the major manifestation of translocated heat injury, it seemed likely that translocated heat injury could be reduced by high humidity. Therefore some plants treated to produce translocated heat injury were placed in moist chambers while controls were placed on the greenhouse bench. Controls showed translocated heat injury in 2 hours whereas plants in the moist chamber did not show translocated injury in 7 hours. When the plants in the moist chamber were placed in the dry greenhouse environment at 7 hours after heating, however, they soon developed translocated heat injury, though less severely than shown by the control plants.

AREA RELATIONS. On the hypothesis that the translocated heat injury was due to a translocated chemical, it would be expected that as the ratio of the area of the heated to the non-heated leaf was increased, translocated injury would be increased. To test this, bean and cowpea plants with approximately equal sized twin leaves were trimmed to relative areas of ¼ : 1, ½ : 1, 1 : 1, 1 : ½, and 1 : ¼, where 1 represents the undisturbed leaf and ¼ and ½ represent the areas of the opposite leaves which were trimmed to these respective fractions of their original areas. In each case the first member of the pair was abraded and the opposite leaf was heated for 5 seconds at 70°. This manipulation gave area ratios of the unheated abraded leaf to the opposite heated leaf of 1 : 4, 1 : 2, 1 : 1, 1 : ½, and 1 : ¼, respectively. In one trial with bean and three trials with cowpea, the average rating for translocated injury in these plants was 5.2, 5.9, 4.4, 2.5, and 1.1, respectively. These results seem to establish that translocated injury decreased as the size ratio of the unheated to the heated leaf increased, and favor the hypothesis that translocated heat injury was due to a translocated chemical.

NO TRANSLOCATED INJURY WITHOUT NECROSIS. If macroscopic translocated injury was due to a translocated toxin, then it might be expected that sublethal dosages of heat would cause injury in sublethal ways such as decreased growth. To test this, one leaf of each of ten beans was heated 10 seconds at 60°, and one leaf of each of ten other plants was detached. This heat dosage was sufficient to kill the heated leaf but not enough to cause macroscopic translocated injury. After 2 weeks the new growth was determined for all plants. No significant difference in the new growth of the two sets of plants was detected, and it is tentatively concluded that translocated heat injury does not occur unless macroscopic symptoms are apparent. However, infection data to be presented later show that heat-induced stimuli could be translocated without macroscopically-apparent translocated injury.

ADDITIVE EFFECT. If direct and translocated heat injury are due to the same chemical, it should be possible to demonstrate injury by sublethal dosages of each type of treatment on the same plant, if the summation of the two amounted to a toxic dose. To test this, one leaf (A) of each cowpea plant was heated for 20 seconds at 60°, which was enough to kill it but usually not enough to cause translocated injury to the opposite leaf. The opposite leaf (B) of plants treated as in A was abraded and heated for 20 seconds at 50° which was usually not enough to cause direct or translocated heat injury. Appropriate controls with the same heat dosages on separate plants were maintained. The B type leaves were more severely injured than were similarly treated leaves on plants of which the opposite leaf was not heated, and more severely injured than were unheated leaves which were opposite to leaves treated for 20 seconds at 60°. It is concluded that an additive injurious effect of direct heat injury and translocated heat injury has been demonstrated.

COMPARISON OF SPECIES. Translocated heat injury has been demonstrated in all species tested, namely: bean, cowpea, peach, cucumber, and *Chenopodium amaranticolor*. Of these bean and cowpea are considered best for experimental work because they have large, opposite, and equal primary leaves. Cowpea is superior to bean in that translocated injury usually occurs on cowpea at lower heat dosages and on younger plants. Bean might be considered superior to cowpea in that the distance of effective translocation is greater in bean. With bean, the distance of effective translocation (length of 2 petioles) may be 2 to 5 inches, whereas with non-petioled primary cowpea leaves, the distance of demonstrated transloca-

tion is much less. Peach and *Chenopodium amaranticolor* have the advantage that they have given as much translocated injury on unabraded as on abraded unheated leaves. From trials with cucumber, peach, and *Chenopodium amaranticolor* it has been demonstrated that injury can be translocated to leaves which are not opposite to the heated leaves.

Wound Healing. When heating and abrasion are widely separated in time, translocated injury still occurs. Results of four tests are given in table I. The heating component of the interaction appeared to disappear first. That is, if heating preceded abrasion, the time from heating until the plants would not respond to abrasion was less than the time until they would not respond to heating if the plants were abraded first. As measured by translocated heat injury, curing of heating injury required about eight hours but curing of abrasion injury required between 18 and 40 hours.

Diurnal Effects. Translocated heat injury occurred fairly regularly from heating and abrasion in the early morning or late evening but irregularly from heating and abrasion in the afternoon. The average rating for translocated injury was 5.1 for treatments between 4 AM and 8 AM (8 trials), 3.9 for treatments between 8 AM and 12 noon (9 trials), 2.4 for treatments between 12 noon and 4 PM (10 trials), 2.2 for treatments between 4 PM and 8 PM (4 trials), and 4.4 for treatments between 8 PM and 12 PM (5 trials).

No Toxin Demonstrated in Vitro. If translocated heat injury is due to some translocated toxic chemical as is suspected, it should be possible to demonstrate such a chemical effect. Limited efforts have failed. When detached plants were placed with their stems in water suspensions of ground heated leaves, or in water with unground heated leaves, there was no specific injury to the detached plants.

Effect of Translocated Heat Injury on Infection. When one of a pair of bean leaves was appropriately heated before or after inoculation of the opposite leaf, infection with TMV was usually greater than on control leaves on comparable unheated plants. Heating varied from 2 to 10 seconds, at 55° to 1 to 4 seconds at 60°. In one trial with heating before inoculation, the average number of TMV lesions on eight control leaves was 3.7, whereas the average number on six leaves opposite heated leaves was 5.3. In six trials with heating after inoculation the average number of lesions on 38 control leaves was 19.7 whereas the average number on 45 leaves opposite heated leaves was 30.3.

With YBV on cowpea the stimulus associated with translocated heat injury was much greater than with TMV on bean. In three trials the average number of virus lesions on eight control leaves was 1.7, the average number on eight leaves of which the opposite leaves were heated 8 seconds at 65° was 5.8, and on eight leaves of which the opposite leaves were heated 16 seconds at 65° was 8.0. This increased infection associated with translocated heat injury occurred despite the fact that the area of living leaf tissue on leaves opposite the heated leaves was less than on the control leaves.

In inoculations with rust, on the other hand, the stimulus associated with translocated heat injury decreased infection. Inoculation with rust was by spraying a uredospore suspension onto the lower surface of bean leaves, and the inoculated leaves were not normally abraded as in inoculation with viruses, and as was usually necessary to produce translocated heat injury. Therefore to test the effect of translocated heat injury on rust infection, an additional control which was abraded on the upper leaf surface was desirable. Also since translocated heat injury was best expressed on plants treated in the early morning.

Table I

Wound Healing & Translocated Heat Injury in Cowpea

1st Treatment	Hr from heating to abrasion	Permanent translocated injury, rating*	Hr from abrasion to heating	Permanent translocated injury, rating
6 AM	0	8	...	...
"	0.5	4	0.5	4
"	2.0	5	2.0	3
"	3.5	0	3.5	1
"	5.0	0	5.0	4
"	8.0	0	8.0	3
"	10.	0	10.	6
2 PM	0	4.5	...	...
"	3.5	6.0	3.5	4.0
"	18.	0	18.	1
"	40.	0	40.	0
4 PM	5.2	1.5	...	...
"	12.	1.5	12.	5.5
9 PM	0	3.5	...	...
"	7.	2.5	7.	3.5
"	16.	0	16.	2.0

* 0 indicates no injury; 10 indicates the leaf was killed; intermediate values indicate proportional injury.

TABLE II

RELATIONSHIP OF TRANSLOCATED HEAT INJURY TO INFECTION BY BEAN RUST

TYPE OF LEAF	AVG. TRANSLOCATED HEAT INJURY (RATING)	AVG. NO. OF RUST LESIONS*
Control, untreated	0	151
Abraded leaves opposite untreated leaves	0	156
Untreated leaves opposite heated** leaves	0	83
Abraded leaves opposite heated leaves	1.6	45

* Average of 14 leaves per treatment.
** 8 or 16 seconds at 65 C before inoculation.

heat treatment and rust inoculations were performed in the early morning, though rust inoculations for most other purposes were usually in the late afternoon. To get rust infection, it was necessary to incubate the plants in a moist chamber for several hours after inoculation, and this is known to reduce translocated heat injury. The average number of rust lesions per inoculated leaf is given in table II.

It is believed that the reduction in numbers of lesions caused by the third and fourth treatments are highly significantly different from the controls and from each other. The reduction on unabraded leaves opposite heated leaves is most interesting because it shows that rust infection detected a translocated inhibitor which was not manifested as visible translocated injury. Abrasion of the upper leaf surface, combined with heat on the opposite leaf, caused an additional reduction in infection which was not apparent from abrasion only. The reduction in infection associated with heat plus abrasion was clearly not largely due to the permanent translocated injury which resulted, because the macroscopic injury occupied a much smaller portion of the leaf than could account for the reduction in infection.

Heating with and without abrasion, as used here, caused a delay in pustule development in addition to the reduction in number of lesions. This delay, estimated as 2 days, was not necessarily associated with apparent injury to the tissue.

One trial of the above type, performed in the early afternoon and without any translocated injury resulting, showed no clear reduction in rust infection. Also when heating and/or abrasion were applied 8 hours after rust inoculation, no reduction in infection was detected.

EFFECTS OF INFECTION ON TRANSLOCATED HEAT INJURY. Rust infection reduces the direct injurious effects of heat (4) and also the translocated injury due to heat (this study). In most of the latter trials, half of each bean leaf on each plant was inoculated with rust and the healthy and rusted halves of the leaf were abraded before the opposite leaf was heated. Translocated injury was consistently less in the rusted tissue than in the normal tissue (fig 1).

DISCUSSION

Translocated heat injury is a common phenomenon in burns of humans (5) but has apparently not been reported with plants. In the present study, translocated heat injury has been demonstrated in five of five plant species tested. With three of these, translocated injury was much greater when the unheated tissue was abraded as in virus inoculation, than when not abraded, but the role of abrasion is not understood.

Translocated heat injury in plants may be comparable to translocation of stimuli by infection (6) and both may be due to translocation of chemicals between twin leaves. However, translocation of chemicals between twin bean leaves is controversial. Rabideau and Burr (4) and Koontz and Biddulph (3) found no translocation between twin bean leaves, but Jacobson and I (9) found translocation of C^{14} and P^{32}. That vascular pathways exist for such translocation is indicated by anatomical work of Doutt (2). The following further evidence supports the idea that translocated heat injury is caused by the same chemicals that are partly responsible for direct heat injury and that these chemicals are translocated from the heated to the non-heated leaves:

I. Except that translocated heat injury usually disappears from the larger veins, translocated and direct injury cause somewhat similar symptoms.

II. Rust infection in bean has been previously shown (7) to reduce direct heat injury, and has been here shown to reduce translocated heat injury.

III. Translocated and direct injury are additive.

IV. Translocated heat injury is increased as the ratio of the area of heated leaf to that of the unheated leaf is increased. That a reciprocal translocation occurred from the non-heated to the heated leaf is not excluded.

This study emphasizes limitations in twin leaf methods of studying physiology and pathology of leaves. Twin leaves are closely comparable for many purposes (1) and are especially useful in many studies of infections, but must be used with caution in studies involving translocation.

The increase in infection of TMV and YBV and the decrease in infection of rust associated with translocated heat injury is correlated with the fact that wound injury is necessary for virus infection, but not necessary for rust infection. This correlation may be accidental. While a translocated stimulus did affect infection with these pathogens, it might be that under different conditions (age of plants, heat dosage, time from heating to inoculation, etc.) there might be a reversed effect, in that a translocated stimulus from heating could cause a reduction in virus infection and an increase in rust infection.

On the basis of this study alone there is no reason to believe that the stimulus associated with translocated heat injury has any practical value in bringing about infections, since with the pathogens studied, heavy infection can be readily induced without heat stimuli.

Summary

When one of the two primary leaves of bean or cowpea was appropriately heated (10 sec at 65 C were suitable) and the opposite leaf abraded, the heated leaf was killed and the opposite leaf was injured. When the heated leaf was removed before the abraded leaf showed injury, translocated injury was prevented. Abrasion of the heated leaf reduced translocated injury but increased direct injury. Abrasion of the non-heated leaf with 1 % K_2HPO_4, or heating the non-abraded leaf in K_2HPO_4 caused less translocated heat injury than abrasion with water or heating in water respectively. Severe translocated injury occurred without abrasion of the non-heated leaves in peach and *Chenopodium amaranticolor* Coste and Reyn. The heat dosage for 50 % translocated heat injury in cowpea was about 140 seconds at 55°, 30 seconds at 60°, 10 seconds at 65°, and 3 seconds at 70°. With beans between 11 and 23 days from seeding, translocated heat injury increased with the age of the plants. Translocated heat injury was reduced by placing the heated plants in a dark moist chamber. As the ratio of the area of the heated leaf to that of the unheated leaf was increased, translocated injury was increased. When translocated injury was not apparent as macroscopic necrosis, there was also no apparent translocated effect of heat on the growth of the opposite leaf or of the growing point. Direct heat and the translocated effect of heat were additive in that sub-injurious dosages of each when used alone could be injurious if applied to different leaves of the same plant. Translocated heat injury could still be demonstrated if abrasion followed heating by as much as 8 hours, or if heating followed abrasion by as much as 18 hours. Translocated heat injury was more severe for treatments in the early morning than for treatments in the early afternoon. Treatments to produce translocated heat injury increased infection of bean by tobacco mosaic virus and of cowpea by peach yellow bud mosaic virus, but decreased infection of bean by rust. Translocated heat injury was reduced in bean tissues previously infected with rust.

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GROWTH & GEOTROPIC CURVATURE OF AVENA COLEOPTILES IN PRESENCE OF *n*-1-NAPHTHYLPHTHALAMIC ACID[1,2]

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INTRODUCTION

The fact that geotropic curvature is mediated by unequal rates of elongation on opposite sides of the bending structure has led to the general implication that depression of these growth rates should cause a corresponding inhibition of geotropic curvature. Recently published results have revealed that this expected relationship was not always observed when plants were treated with compounds such as 2,3,6-trichlorobenzoic acid and *n*-1-naphthylphthalamic acid (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12). Selective inhibition of geotropic curvature by *n*-1-naphthylphthalamic acid (NP) has been reported for a number of plants, including seedlings of corn, crucifer, cucumber, pea, lentil, sunflower, and tomato (3, 4, 5, 8). Since the data of Tsou Ching, Hamilton, and Bandurski (8) did not reveal a differential effect of NP on growth and geotropic curvature of intact Avena coleoptiles, the present study was undertaken to explore, in additional detail, the effects of NP on elongation and negative geotropism of isolated apical segments of Avena coleoptiles.

MATERIALS & METHODS

Avena sativa L. seeds, Victory Strain (U.S. Dept. of Agriculture, C. I. 2020)[3] were used in these experiments. The seedlings were grown on filter paper strips immersed in distilled water which previously had been aerated. Additional details of the growing method are described elsewhere (11). Only seedlings that were 72 hours old and that had 30 mm (±2mm) coleoptiles were used.

Growth measurements were made by two methods. In one group of experiments the coleoptiles were isolated from the seeds and primary leaves. The apical 5 mm sections were discarded; the second 5 mm sections were used. These sections were floated in petri dishes containing the media (20 sections/20 ml solution). All sections were randomized in distilled water and then floated on the experimental media. During cutting and transferring, the sections were exposed to red neon light (wave lengths longer than 6,074 A). The sections were allowed to elongate in the dark for 24 hours. At the end of this period, their lengths were measured with a micrometer in the ocular of a dissecting microscope.

In the other growth measurements, apical 15 mm coleoptile sections were used. These sections had intact apices, but the leaves were pulled out. Six such sections were placed in each of two racks which held them in the upright position and exposed only the cut basal ends to the medium. Both racks were placed in a transparent chamber to maintain high humidity. The sections were photographed in flashes of red light (wave lengths above 6,000 A) at 30 minute intervals with a time-lapse apparatus. Measurements of lengths were made from the negative. The basal medium contained only aerated distilled water and the indicated concentrations of NP.

Curvature measurements also were made on apical 15 mm coleoptile sections. These were isolated and mounted on sections of stainless steel needles with stops 5 mm from one end. The individual coleoptile holders were fitted into lucite block containers (6). Only the cut basal ends of the coleoptiles were exposed to the aerated distilled water or other solutions. The coleoptiles were left for 2 hours in the holders in upright position before they were geotropically stimulated. After geotropic exposure and curvature development time, the angles of curvature were measured from negatives which were made by shadowgraphing. The entire procedures were carried out at a temperature of 22 C (±1°).

Commercial NP was purified and recrystallized according to the method outlined by Tsou Ching et al. (8). (The details of this procedure were kindly supplied in a personal communication from Dr. R. S. Bandurski). The recrystallized material (mp 193 C) had a molar extinction coefficient of $E_{284} = 0.92 \times 10^4$.

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[3] Supplied by the U.S. Department of Agriculture.

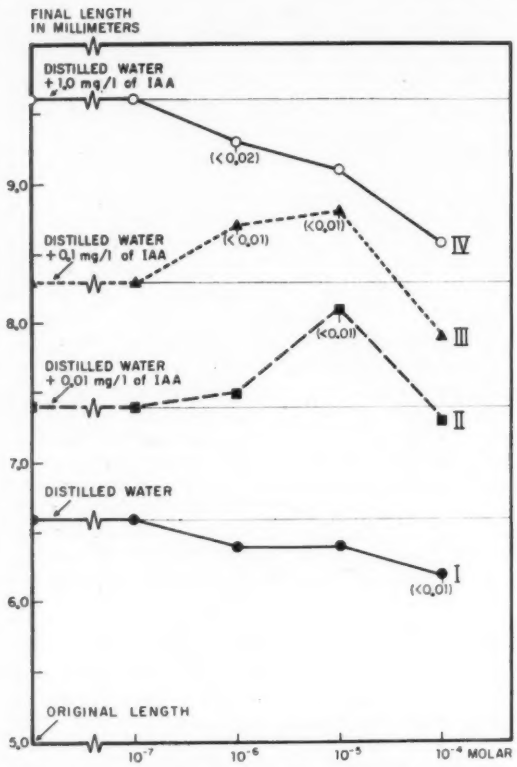

FIG. 1. Effects of various concentrations of NP on 5 mm floating Avena sections. Each point represents an average of 40 or more sections. Measurements of length were made after 24 hours of growth. Curve I represents elongation of sections in aerated distilled water plus NP. For curves II, III, and IV, the growth medium contained the indicated concentrations of IAA plus NP in aerated distilled water. The numbers in parenthesis directly under certain points in the curves are probability values which indicate the level of significance when these points are compared to their control by the "t" test.

RESULTS

SECTION ELONGATION. As shown by curve I in figure 1, NP, in concentrations of 1×10^{-5} M and less, had no significant effect on the length of 5 mm floating Avena sections which were measured after 24 hours of growth. A slight inhibition of elongation was caused by 1×10^{-4} M NP. Since NP did not stimulate growth of oat sections in the absence of auxin, it should not be classed as an auxin for this tissue. This observation is in agreement with previous findings (8). Under the conditions of the present experiments, the effects of NP were unlike those caused by 2,3,6-trichlorobenzoic acid (TCBA) which accelerated growth at concentrations of 1×10^{-4} M or less (6, 7).

In the presence of exogenous IAA, 0.01 mg/l, 1×10^{-5} M NP caused a significant increase in the growth of floating sections. When 0.1 mg/l of IAA was present in the medium, 1×10^{-5} and 1×10^{-6} M NP stimulated growth. (Curves II & III in fig 1.) This slight stimulatory effect was not reported by Tsou Ching et al. (8). When 1 mg/l of IAA was added to the medium (curve IV), concentrations of NP above 1×10^{-7} M inhibited growth; the inhibition became more effective with increasing concentrations of NP. This fact lends support to the previous concept of competitive interaction between NP and IAA when both were used at higher concentrations (8).

Time-lapse growth curves of 15 mm isolated coleoptiles with intact apices are shown (fig 2). The control curve practically duplicates previously published growth rates for sections of this kind (6). Inhibition of elongation by 1×10^{-4} M NP became apparent after 6 hours. This inhibition was consistent with the effect of 1×10^{-4} M NP on floating sections as shown in the previous figure. Thus high concentrations of NP are inhibitory to coleoptile elongation in the presence of endogenous as well as exogenous auxin when relatively long growth periods were used. This concentration of NP also has been demonstrated to be inhibitory to growth of other plants (8). However, the magnitude of the inhibition of oat coleoptile growth by 1×10^{-4} M NP (fig 2) is not as extensive as inhibition reported by previous investigators (1, 8). Although the data are not included in figure 2, 1×10^{-6} and 1×10^{-7} M NP had no effect on the elongation of 15 mm apical sections.

As might be anticipated (fig 1), 1×10^{-5} M NP proved to increase elongation of apical sections during 24 hours of growth. (See curves in fig 2.) The results shown in figure 2A were obtained by making measurements during the last 3 hours of a 5 hour period of exposure to NP. Only the cut basal ends of the isolated coleoptiles were exposed to the solutions. Time-lapse photographs were made at 15 minute intervals. The growth during these 3 hours is particularly important in relation to the succeeding curvature data. Curves showing the effects of 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} M NP were omitted from figure 2A because the growth curves for these three concentrations of NP essentially duplicated the control curve. It is evident, however, that 1×10^{-4} M NP is stimulatory to elongation at least during the first hours of growth. (The curves in fig 2A show more growth than the curves in fig 2, because the latter were not pretreated with NP or rested in control solutions for 2 hr prior to the initiation of measurements.)

GEOTROPIC CURVATURE. From previous studies it has been demonstrated that TCBA had differential effects on elongation and geotropic bending of Avena coleoptiles (6, 7, 9). The influences of several concentrations of NP on negative curvature are shown in figure 3. The coleoptiles were isolated and their cut basal ends were exposed to the NP solutions for 2 hours before initiating geotropic stimulation. NP

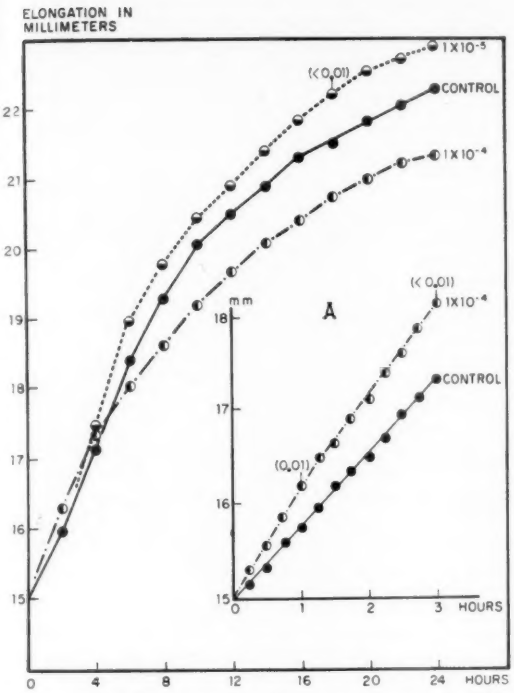

FIG. 2. Growth curves of 15 mm Avena coleoptile sections with intact apices showing the effects of various concentrations of NP. Each point represents an average of 18 or more values. FIG. 2A (*insct*). Growth of similar apical sections during the last 3 hours of a 5 hour exposure to NP. Each curve represents an average of 18 or more values. The numbers in parenthesis directly above certain points in the curves are probability values which indicate the level of significance when these points are compared to their control by the "t" test.

(1×10^{-7} M) proved stimulatory to curvature for all geotropic exposure times used; 1×10^{-6} M was stimulatory for exposures up to 90 minutes. These concentrations had no significant effect on elongation of sections with intact apices, but 1×10^{-6} M increased the growth of floating sections in the presence of 0.1 mg/l of IAA (curve III in fig 2). It is possible that this stimulation of curvature was brought about by a slight acceleration of growth by NP, which was amplified by the differential elongation of opposite sides of the bending coleoptiles.

For horizontal exposures longer than 30 minutes, 1×10^{-5} M NP suppressed geotropic bending. The same concentration increased the elongation of 5 mm floating sections and of 15 mm apical segments. Thus, under these conditions, NP had opposite effects on growth and geotropic curvature. A tenfold higher concentration of NP had similar but more extensive effects. After 5 hours of exposure to NP, elongation was increased by about five percent (fig 2A), while geotropic curvature after 2 hours of pretreatment plus 3 hours in the horizontal position was decreased by 70 %. (See fig 3.)

To further analyze the effects of NP, geotropic curvatures also were measured after geotropic stimulation plus 30 minutes of development time on a horizontal klinostat rotating at 1 rpm (fig 4). (All points are plotted as total time lapsing between the initiation of geotropic stimulation and the final curvature.) Curves I, II, III, and IV are duplicates of the first portions of curves presented in figure 3. These coleoptiles remained in the horizontal position for the durations indicated on the abscissa. Except for the first point in curve I-K, the similarity of curve I and curve I-K shows that geotropic stimulation for 60 and 90 minutes, plus an additional 30 minutes of development time on the klinostat, induced essentially the same curvatures as continued stimulation of 90 and 120 minutes, respectively. Geotropic stimulation for 30 minutes plus 30 minutes of development on the klinostat, resulted in more curvature than 60 minutes of stimulation in the horizontal position ($p = < 0.01$). Thus, when the duration of stimulation was as much as 60 minutes, an additional 30 minutes on the klinostat permitted the development of essentially the same curvature as an extra 30 minutes in the horizontal position.

In the presence of all concentrations of NP used (1×10^{-4}, 1×10^{-5}, & 1×10^{-6} M), the sum of the stimulation time plus klinostat time induced less geotropic bending than the equivalent total duration in the horizontal position. (See fig 4.) When NP was not present, 30 minutes on the klinostat subsequent to geotropic stimulation induced curvature at least as efficiently as an additional 30 minutes in the horizontal position. (Compare curves I-K & I.) In the light of these observations it becomes relevant to inquire whether the NP exerts its inhibitory effect during the period of geotropic stimulation or during the development time on the klinostat. Preceding results have shown that 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} M NP had no inhibitory effect on elongation of apical coleoptile segments. This relationship implies that 1×10^{-5} and 1×10^{-6} M NP must be exerting the inhibitory effect on the coleoptiles while they are in the horizontal position.

The curves in figure 2A show that 1×10^{-4} M NP stimulated apical segment elongation. As shown by the curves in figure 4, 30 minutes of supplementary klinostat development again resulted in less curvature than an additional 30 minutes in the horizontal position. In combination, these facts also indicate that NP must be exerting its inhibitory effect during the period of geotropic stimulation.

If the curvatures (fig 4) were plotted against duration in the horizontal position—this has the effect of moving each point on the K curves to the left by the distance equal to 30 minutes—several other considerations become apparent. Table I shows this effect in a more practical way. Thirty minutes of klinostat development of coleoptiles in distilled water

TABLE I

MIN IN HORIZONTAL POSITION	MIN ON KLINOSTAT	° OF CURVATURE			
		DISTILLED WATER	1×10^{-6} M NP	1×10^{-5} M NP	1×10^{-4} M NP
30	0	10	16	14	8
30	30	30	25	18	10
60	0	25	32	22	13
60	30	40	32	25	12
90	0	42	37	30	16
90	30	56	40	32	16

Geotropic curvatures of Avena coleoptiles in the presence of the indicated concentrations of NP.

gave more extensive curvature for all durations of stimulation. In the presence of NP, only 1×10^{-6} and possibly 1×10^{-5} M, gave increased curvatures when klinostat development followed 30 minutes of geotropic stimulation. This could be due to an acceleration of growth by these concentrations of NP. Such increase in growth was observed with floating sections in the presence of exogenous IAA, although these measurements were made at the end of a 24 hour period.

Klinostatic development with all of the indicated concentrations of NP had no significant effect on upward curvature when the stimulation time was 60 or 90 minutes. These facts substantiate the position that NP must be mediating its inhibitory effect during the period of geotropic stimulation.

PHOTOTROPIC CURVATURE. Results in a previous paper (6) revealed that the several concentrations of TCBA which inhibited geotropism had no effect

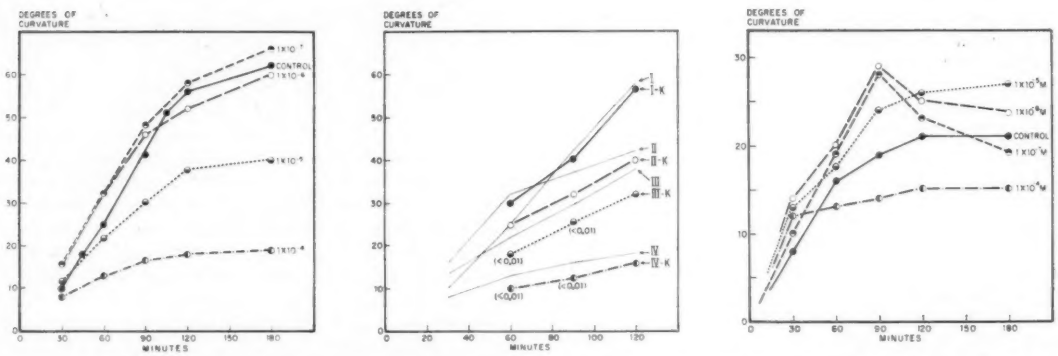

FIG. 3. The effect of NP on geotropic curvature of 15 mm apical Avena sections measured at the end of the indicated durations in the horizontal position. Each point represents an average of 40 or more values. A difference of 7° between corresponding points in two curves gives probability values less than 0.01.

FIG. 4. Geotropic curvatures of 15 mm apical Avena coleoptile sections showing the effects of various concentrations of NP. Duplicates of curves I, II, III, and IV in figure 3 are repeated for comparison. For these curves the numbers on the abscissa indicate minutes in the horizontal position. Each point on the curves labeled K represents an average of 40 or more measurements from coleoptiles that were developed on the klinostat for 30 minutes after geotropic stimulation. For these curves the times indicated on the abscissa represent geotropic stimulation plus 30 minutes of klinostat time. Curve I-K. Geotropic curvatures of control sections in aerated distilled water. Curves II-K, III-K, and IV-K are from coleoptiles treated with 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} M NP, respectively. The numbers in parenthesis directly under certain points in the curves are probability values which indicate the level of significance when these points are compared to their control by the "t" test.

FIG. 5. The effect of NP on positive phototropic curvature of 15 mm apical Avena sections measured at the indicated times after stimulation. Each point represents an average of 40 or more values. A difference of 5° between corresponding points in two curves gives probability values of less than 0.01 when calculated by the "t" test.

on the positive phototropic curvature of apical coleoptile segments. (See also 9.) The curves (fig 5) show that effects of NP on phototropic bending are unlike the effects of TCBA. All of the concentrations of NP that were used induced a slight increase in bending 30 minutes after phototropic stimulation (all coleoptiles were exposed to 0.2 ft-c of unilateral white light for 125 sec). Ninety minutes after stimulation by unilateral light, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} M NP induced a much larger curvature. For the two lower concentrations, the maximum curvature was attained in 90 minutes, after which the curvatures decreased rather rapidly. Although direct evidence is lacking, it is possible that the increased curvature could be mediated by an effect of NP on elongation.

Coleoptiles treated with 1×10^{-4} M NP manifested inhibition 90 minutes or more after stimulation. There was no difference in the magnitude of curvature at 120 and 180 minutes after stimulation. NP (1×10^{-4} M) also effectively inhibited geotropic bending. Since this concentration of NP was stimulatory to growth (fig 2A), it is difficult to anticipate the nature of the NP inhibited mechanism which is involved in phototropic curvature.

Discussion

In previous studies 2,3,6-trichlorobenzoic acid had dissimilar effects on elongation and geotropic curvature (6, 7). The available data indicated that TCBA was probably exerting its effects by suppressing the geotropic perception mechanism. Selective inhibition of geotropic curvature by NP has been reported for several different seedlings but not for intact Avena seedlings (8). My results show that 1×10^{-4} M NP stimulates elongation of 15 mm apical coleoptile segments and inhibits their negative geotropic bending.

On the basis of the results presented in this paper, it is not justifiable to classify NP as an ordinary auxin. While some concentrations of NP stimulated Avena coleoptile elongation (see figs 1 & 2) they did so only in the presence of low concentrations of IAA or in combination with endogenous auxin. In the presence of endogenous auxin in 15 mm apical segments, NP (1×10^{-4} M) induced stimulation of elongation only for the first 6 hours of growth, then it became inhibitory. This combination of effects, caused by a single concentration of a compound, has not been accepted as a general characteristic of auxins.

The slight increase in geotropic curvature and the relatively large stimulation of phototropic curvature caused by the lower concentrations of NP, tentatively can be attributed to the increase in the elongation caused by the NP. In this connection, it was again noted that the stimulation effect of 1×10^{-6} M for geotropism and 1×10^{-6} and 1×10^{-7} M NP for phototropism was most evident in responses of shorter durations.

From results of a previous study (6) it has been demonstrated that certain concentrations of TCBA had differential effects on growth and geotropic bending of Avena coleoptiles. The same concentrations of TCBA did not alter phototropic curvature. In this combination NP mediated somewhat different effects. NP (1×10^{-4} M), which was inhibitory to geotropic bending and stimulatory to elongation, also depressed bending toward unilateral light. These facts, plus the observation that NP does not function as a typical auxin, support the conclusion that NP and TCBA are probably affecting different mechanisms.

It is indicated from the results herein reported, that higher concentrations of NP must inhibit tropic bending by influencing some process other than the elongation mechanism. Results of the experiments, in which geotropic stimulation was followed by klinostatic development, implied that NP mediated its inhibition during the period of stimulation and not during the subsequent development on the klinostat. Additional study is required for a more precise characterization of the mechanisms affected by the NP.

Summary

I. I measured the effects of 1×10^{-7} M to 1×10^{-4} M NP on the growth of 5 mm floating *Avena sativa* L. sections, on elongation of 15 mm apical segments and on geotropism and phototropism of apical segments.

II. Growth of floating sections was increased by 1×10^{-6} and 1×10^{-5} M NP, but only in the presence of exogenous IAA. NP (1×10^{-4} M) was slightly inhibitory to growth of floating sections in the presence or absence of IAA.

III. NP (1×10^{-5} M) induced a slight stimulation of elongation of 15 mm apical coleoptile segments for the entire 24 hour growth period. Higher concentrations of NP (1×10^{-4} M) caused increased elongation during the first 6 hours of exposure and inhibition for longer growth periods. Two concentrations of NP (1×10^{-6} & 1×10^{-7} M) had no effect on apical segment elongation.

IV. Both 1×10^{-4} M and 1×10^{-5} M NP were inhibitory to negative geotropic curvature of 15 mm apical Avena segments, while 1×10^{-6} M and 1×10^{-7} M caused slight stimulation of bending for stimulation times up to 90 minutes. Experiments utilizing klinostatic development time subsequent to geotropic stimulation, indicated that NP treated coleoptiles attained smaller curvatures than untreated segments.

V. Positive phototropism, in response to 270 meter-candle-seconds of unilateral illumination, also was inhibited by 1×10^{-4} M NP. All other concentrations of NP induced stimulation of phototropic bending 90 minutes after application of the light.

For 1×10^{-6} M and 1×10^{-7} M, the stimulatory effect was not evident 180 minutes after exposure to light.

VI. I interpreted these results to indicate that NP did not function as a typical auxin and that negative geotropism of Avena coleoptiles was inhibited by NP by exerting its influence during the period of stimulation in the horizontal position.

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RED, FAR-RED RESPONSE & CHLOROPHYLL SYNTHESIS[1,2,3]

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One of the earliest observations on the photomorphogenic development of chlorophyll synthesis was made by Withrow et al. (10). In a preliminary report they noted that pretreatment of leaves with up to 10 millijoules (mj/cm²) of red radiant energy followed by a dark period of from 5 to 15 hours, resulted in the elimination of the latent period in subsequent chlorophyll formation, with some indication that the red induction might be reversed by an exposure to far-red. Virgin verified this observation of the involvement of the photomorphogenic induction effect on the development of the chlorophyll synthesizing mechanism (6) and demonstrated the greater effectiveness of red over blue pretreatment (7). Although the induction response has been clearly demonstrated, the far-red reversal phenomenon associated with chlorophyll synthesis has not been previously shown. The data herein presented establish clearly that chlorophyll synthesis is another of the physiological responses referred to as red, far-red reactions, in which stimulation of plastid pigment synthesis by a short red preirradiation may be nullified by a subsequent far-red pretreatment. However, the red, far-red effect on chlorophyll synthesis is not directly associated with the photoconversion of protochlorophyllide (11). It is, rather, a reflection of metabolic and growth changes affecting the rate of synthesis of protochlorophyllide, these growth and metabolic changes having been induced by means of the primary effect of light on the photomorphogenic pigment receptor (4).

METHODS

Bean plants (*Phaseolus vulgaris* Linn. var. Burpee's stringless green pod) were used in all experiments. The seeds were planted in gravel beds subirrigated with tap water. The tops of 6-day-old etiolated seedlings were excised just below the cotyledons. One cotyledon was removed and the remaining pair of primary leaves with the attached cotyledon was placed leaves up in a petri dish containing water-moistened filter paper; 25 plants were used for each sample. The preparation and handling of tissue were carried out as quickly as possible under a green safelight (9). No detectable responses were induced by the safelight exposure. Temperature was maintained at 25 C.

Immediately following the harvest and preparation of leaf samples, the samples were pretreated by exposure to saturating levels of red or far-red, or both. The source of energy for the red induction was a red fluorescent tube luminaire and a red gelatin filter. The induction exposure was 3 minutes at an irradiance of 40 μw/cm² (7 mj). Interference filters and a projection lamp were used to obtain monochromatic energy at 730 mμ for the far-red pretreatment (8). One hundred mj/cm² of far-red were administered in 5 minutes. Upon completion of the pretreatments, the samples were placed in the dark for an 18-hour development period.

To determine the effects of pretreatments on the rate of subsequent chlorophyll synthesis, the continued formation of chlorophyll over extended periods of time was measured by exposing samples to white light (1,000 μw/cm²) from a luminaire of warm white fluorescent tubes. In the time course studies, illumination was continued for various periods up to 8 hours. In the later experiments, a continuous exposure to 3 hours of high intensity white light was used as an assay period. One of a pair of samples was removed after 5 minutes, whereas the other sample remained under the luminaire for the entire 3-hour period. The net difference in chlorophyll synthesized between these two samples represented newly synthesized chlorophyll and, therefore, reflected any changes in the rate of protochlorophyllide synthesis resulting from earlier pretreatments of red and far-red radiant energy. The procedural steps in the preparation and treatment of leaf samples were as follows:

HARVEST PRETREATMENT

Red

40 μw/cm² — 3 min

Far-Red

300 μw/cm² — 5 min

18 *Hr dark development period*

White light assay

1,000 μw/cm² — 5 min; 3 hr

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TABLE I

TIME COURSE OF SUBSEQUENT CHLOROPHYLL* SYNTHESIS AS AFFECTED BY RED (R) & FAR-RED (FR) PRETREATMENT

WHITE LIGHT ASSAY TIME	PRETREATMENT				NET GAIN OVER FR	
	DARK	FR	R	R, FR	R	R, FR
5 min	11	11	13	13	2	2
1 hr	28	28	49	39	21	11
2	53	63	105	70	42	7
3	91	102	199	112	97	10
5	213	245	348	248	103	3
6	279	304	407	297	103	−7
7	280	331	486	340	155	9
8	344	404	579	433	175	29

* μg/g Fresh weight

Chlorophyll content was measured spectrophotometrically in an aqueous acetone extract of macerated boiled leaf tissue and was calculated in μg/g fresh weight using the absorption coefficients given by Arnon (1).

RESULTS & DISCUSSION

The effect of a red or far-red, or a red followed by far-red pretreatment, can best be evaluated by following the synthesis of chlorophyll over an extended period of time. The development of chlorophyll during an 8-hour exposure to white light of leaves that had been subjected to these pretreatments is presented in table I. Samples were pretreated immediately after harvest, developed in the dark for 18 hours and finally placed under the white light luminaire. During the 8-hour assay exposure, samples were periodically removed for chlorophyll determinations. One observation of significance is that the far-red pretreatment itself does have some induction effect, conceivably owing to the overlapping of the red induction and far-red reversal action, in the spectral region transmitted by the far-red monochromator. Compared with either the untreated or the far-red samples as a control, the gain by a red induction pretreatment is marked. The net gain of the red samples and of the resultant response to red plus far-red exposure, over that of the far-red pretreatment, reveals a high degree of efficiency for the far-red reversal system.

Downs (2) and Liverman (5) have shown that after several alternating exposures to red and far-red, the quality of the terminal treatment dominated the final response in leaf elongation or leaf disk expansion. Data (table II) from similar experiments indicate that chlorophyll synthesis is likewise affected. Leaves with attached cotyledons were pretreated alternately with a 3-minute red and 5-minute far-red exposure, returned to the dark for 18 hours, to be followed by 3 hours of white light. Chlorophyll accumulation during the first 5 minutes of the white light assay period represented that protochlorophyllide converted by the initial pretreatments, plus the conversion of the additional protochlorophyllide synthesized during the 18-hour dark period. The net difference between pigment developed in 3 hours and that formed in 5 minutes reflects the influence of the preirradiation on the subsequent rate of protochlorophyllide synthesis. From the data, it is apparent that the final light pretreatment determines the level of response by the pigment-synthesizing mechanism. In each case where several cycles of red followed by far-red were administered, the capacity to synthesize chlorophyll was reduced to a level approaching that of the net effect of the successively applied far-red exposures.

In order to determine the effectiveness of the photoinactivation by far-red with relation to time, a dark period of varied duration was interposed between the red and the far-red pretreatments. Table III delineates the effect of such an interposed period of darkness. The samples were first treated with red light, then subjected to a dark time of varied duration up to 9 hours, after which the far-red was applied. All samples were then returned to the dark overnight, and the next morning one of each pair of samples was exposed to 5 minutes of white light, while the second

TABLE II

EFFECTS OF RED (R) & FAR-RED (FR) RADIANT ENERGY ON SUBSEQUENT CHLOROPHYLL* SYNTHESIS

PRETREATMENT	WHITE LIGHT ASSAY		Δ (3 hr–5 min)
	5 min	3 hr	
Dark	13	84	71
FR	14	89	75
R	15	191	176
R,FR	17	106	89
R,R	18	186	168
R,FR,R	18	208	190
FR,FR	14	97	83
R,FR,R,FR	22	120	98
R,R,R	22	209	187
R,FR,R,FR,R	24	215	191
FR,FR,FR	15	104	89
R,FR,R,FR,R,FR	28	128	100

* μg/g Fresh weight

TABLE III

EFFECT OF DARK PERIOD INTERPOSED BETWEEN RED (R) & FAR-RED (FR) PRETREATMENTS ON SUBSEQUENT CHLOROPHYLL* SYNTHESIS

PRETREATMENT			WHITE LIGHT ASSAY		△ (3 hr–5 min)	NET GAIN OVER FR	% REVERSAL
RED	DARK TIME (hr)	FR	5 min	3 hr			
...	0	...	9	103	94	...	...
R	0	...	11	216	205	95	...
...	0	FR	10	120	110	0	...
R	0	FR	12	130	118	8	92
R	½	FR	11	129	118	8	92
R	1	FR	11	126	115	5	95
R	2	FR	11	140	129	19	80
R	3	FR	11	141	130	20	79
R	5	FR	12	152	140	30	68
R	7	FR	11	133	122	12	87
R	9	FR	10	143	133	23	76

* μg/g Fresh weight

sample received 3 hours of white light. The data indicate that the red induction effect on chlorophyll synthesis is reversible even after a lapse of as much as 9 hours between the application of the induction and reversal energies. During the extended interposed dark time, the loss of reversibility was less than 25%. This loss with time, in the photoinactivating ability of the far-red, is in agreement with the observations on plumular hook response (3) and leaf expansion (2) made by others, although the relative decrease in chlorophyll synthesis with dark time is appreciably less than the decrease in response in the morphological reactions.

Many aspects of the interrelationship of photomorphogenesis and plastid pigment synthesis are in need of elucidation. More specifically, it remains to be determined whether the stimulatory effect is on endo- or exoplastid biochemical systems, particularly in the synthesis of pigment precursor, and whether or not the radiant energy pretreatments finally manifest themselves in altered plastid maturation and/or in number.

SUMMARY

The data characterize the development of the chlorophyll synthesizing mechanism as one of the many morphological and physiological responses subject to the mediation of the red, far-red photoreceptor. The rate of chlorophyll synthesis in etiolated leaves can be influenced by a short preirradiation with red and far-red radiant energy. The induced stimulatory effect of a red pretreatment can be nullified by a subsequent exposure to far-red administered either immediately after the red induction exposure or even after the interposition of several hours of darkness. When red and far-red are administered alternately for several cycles, the quality of the terminal treatment determines the level of response.

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EFFECT OF IRON CHLOROSIS ON PROTEIN FRACTIONS OF CORN LEAF TISSUE [1, 2]

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Many concepts of the physiological changes in plants, as related to the organic and inorganic constituents, were determined by bulk analysis. Today a galaxy of new physical and chemical techniques is available to isolate and study individual components of cell tissue, thus permitting far more detailed work.

During the last few years the biochemistry, synthesis, and metabolism of plant proteins have been extensively investigated because of their association with enzymes and the fundamental role these molecules play in most biological phenomena (5, 9, 10, 11, 12, 14, 15, 16). The leaf is a seat of enzyme activity and leaf proteins are undoubtedly involved in a major way in photosynthetic reactions. The location of any protein relative to the architecture of the cell, and its separation in pure form from the heterogeneous substances making up the leaf, present extraction problems. Nevertheless, there is much interest in the physical, chemical, and enzymatic properties of cytoplasmic proteins as well as leaf proteins that are thought to be associated with enzymes in different particulate components inside the cell.

The present investigation considers the effect of iron chlorosis on the pattern of protein distribution in corn leaf tissue.

EXPERIMENTAL DETAILS & METHODS

After 7 days in a germinator, corn plants (*Zea mays* L.) were transplanted to Hoagland's No. 2 nutrient solution (6) and grown under controlled conditions of light intensity and temperature. The solutions were adjusted daily to pH 5.5, aerated continuously, and changed every 4 days. Seven days after transplanting, one treatment group received complete nutrients while the other received all nutrients except iron. Each treatment group consisted of five jars with two plants in each, all of which constituted one replication. A total of six replications was run. Leaves from ten plants (5 × 2) were sampled in each treatment per replication.

Three weeks after transplanting, or two weeks after treatment was begun, the plants were harvested. At this stage the main difference between the two groups of plants was the chlorosis in the minus iron treatment. For analysis the plants were taken to a cold room and the second, third, and fourth leaves from the top were harvested and pooled. Any surface dust was wiped off with a damp sponge. Along with the midrib, 4-inch portions from both the tip and the base of the leaves were discarded, and the remaining parts were torn into half inch segments. From this composited leaf material a 25 g sample was preserved in a deep freeze for fractionation analysis. Nitrogen and protein determinations were made on subsamples of the remaining fresh leaf tissue by the Kjeldahl (1) and folin phenol reagent (11) methods while phosphorus and iron were determined on a wet digestion of the dry plant tissue (8).

For ease of homogenizing, each sample was divided at the time of analysis by weighing into two equal portions. The tissue was allowed to thaw at room temperature for 15 minutes, taken to the cold room, and then transferred to a chilled stainless steel jar (200 ml) lined with teflon. The tissue was allowed to soak in 50 ml water for 5 minutes and then homogenized for 1½ minutes in an omnimixer (Servall model OM) operated at full speed. The jar was kept in a crushed ice bath to prevent a rise in temperature during homogenization. The resulting slurry was filtered through four folds of cheese cloth.

The homogenates from the two portions were mixed and subjected to fractionation by differential centrifugation in a cold room. Relative centrifugal forces up to 17,000 × *g* were obtained using nylon 50 ml tubes in a Servall type SS-34 super-speed centrifuge with remote speed control. For higher centrifugal forces, the refrigerated Spinco model L ultra centrifuge with rotor head No. 40 and 10 ml celluloid tubes were used. The scheme of fractionation procedure as well as the various particles identified under a phase microscope is summarized in figure 1.

After spinning the homogenate at the appropriate speed, the supernatant was transferred by means of a mechanical pipette to another tube for successive centrifugation. Each sediment was washed once with water and recentrifuged, and the wash then transferred to the succeeding fraction. The pellets obtained in different fractions were resuspended in water by homogenizing in a Ten Broeck homogenizer, and the final volume was made to 50 ml. The clear supernatant obtained in the last fraction was made to 150 ml.

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RESULTS & DISCUSSION

The total nitrogen and protein contents of the two sets of leaf samples are given in table I. There was no difference between the nitrogen contents of green and chlorotic leaves. The Kjeldahl method includes both protein and non-protein nitrogen. In contrast with nitrogen, the protein level for chlorotic leaves was 25 % below that of normal leaves.

Preparations from the various fractions were examined under a phase microscope using an oil-immersion lens. For this purpose the sediments in different fractions were suspended in water by gentle shaking. The particles in the prepared suspensions are described in figure 1.

The results for proteins in this experiment are much the same as for the corresponding fractions of McClendon's work (12). Although in the present investigation exactly the same centrifugal forces as those published by Gordon (5) were employed, results from the two studies do not agree except in the last fractions. This may be attributed to the difference in density of mediums used. Gordon (5) used 0.2 M phosphate buffer in 0.3 M sucrose solution.

No reference was available with which to compare the effect of iron chlorosis on the pattern of protein fractions in leaf tissue.

The cytoplasmic or soluble proteins did not change much with iron deficiency. It is suggested that further fractionation of this fraction by electrophoresis may show differences.

Bonner (3) has reported that chloroplastic proteins accounted for roughly 40 % of the total nitrogen in spinach leaves; our reduced protein levels in corn leaves are correlated with a reduction in chloroplasts. The iron that is active in chlorophyll formation is actually bound in organic combination in the chloroplasts and is essential for initiating synthesis of chloroplastic proteins in leaves (2, 9). Iron deficiency has therefore suppressed the protein synthesis in chlorotic leaves. However, nitrogen absorption continued in spite of low demand in the leaves for protein synthesis and resulted in the accumulation of non-protein nitrogen in chlorotic leaves, as indicated by a low protein to nitrogen ratio in these leaves when compared with the normal ones. Similar observations have been made by other investigators (2, 4, 7, 13). Bennett (2) reported that iron deficiency leads to an increase in amino acid nitrogen and a decrease in protein nitrogen. DeKock and Morrison (4) correlated free amino acids with phosphorus/iron (P/Fe) ratio in leaves; the acids are high when the ratio is high and decline as the ratio declines. This, they say, was true irrespective of the cause of chlorosis. In the present experiment also, the P/Fe ratio was higher in chlorotic than in green leaves (table I). This might have contributed to the higher concentration of non-protein nitrogen in chlorotic leaves.

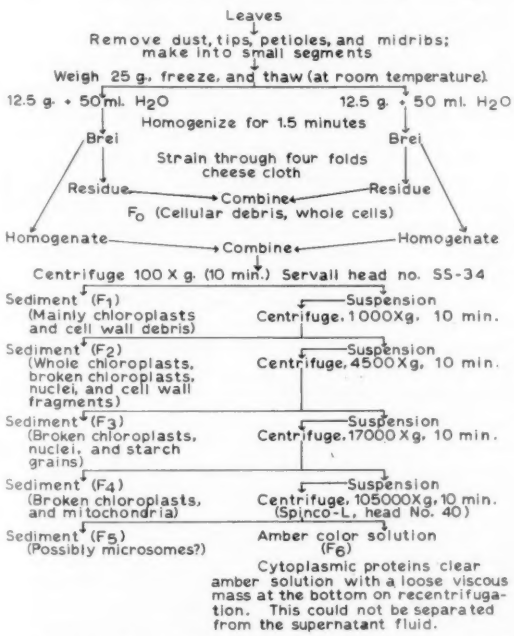
Leaves
Remove dust, tips, petioles, and midribs; make into small segments
Weigh 25 g., freeze, and thaw (at room temperature)
12.5 g. + 50 ml. H2O
12.5 g. + 50 ml. H2O
Homogenize for 1.5 minutes
Brei
Brei
Strain through four folds cheese cloth
Residue
Residue
Combine
F0 (Cellular debris, whole cells)
Homogenate
Combine
Homogenate
Centrifuge 100 X g. (10 min.) Servall head no. SS-34
Sediment (F1) (Mainly chloroplasts and cell wall debris)
Suspension Centrifuge, 1000Xg, 10 min.
Sediment (F2) (Whole chloroplasts, broken chloroplasts, nuclei, and cell wall fragments)
Suspension Centrifuge, 4500Xg, 10 min.
Sediment (F3) (Broken chloroplasts, nuclei, and starch grains)
Suspension Centrifuge, 17000Xg, 10 min.
Sediment (F4) (Broken chloroplasts, and mitochondria)
Suspension Centrifuge, 105000Xg, 10 min. (Spinco-L, head No. 40)
Sediment (F5) (Possibly microsomes?)
Amber color solution (Fe)
Cytoplasmic proteins clear amber solution with a loose viscous mass at the bottom on recentrifugation. This could not be separated from the supernatant fluid.

FIG. 1. Flow sheet for fractionation of corn leaf proteins.

TABLE I

NITROGEN, PROTEIN, PHOSPHORUS, & IRON IN NORMAL & CHLOROTIC CORN LEAVES

REP. NO.	PROTEIN	N	PROT./N	P	Fe	P/Fe
	% *on dry wt*			meq/100g *dry wt*		
			Green leaves			
1	29.5	4.3	6.9	4.9	0.23	21
2	34.2	4.5	7.6	4.5	0.22	20
3	29.0	4.4	6.6	4.2	0.20	21
4	32.4	4.2	7.7	5.2	0.25	21
5	32.4	4.3	7.5	4.3	0.23	19
Mean	31.5	4.3	7.3	4.6	0.23	20
			Chlorotic leaves			
1	21.1	3.9	5.4	3.4	0.02	170
2	22.8	4.1	5.6	3.4	0.02	170
3	25.2	4.2	6.0	3.3	0.02	165
4	23.7	4.1	5.8	2.6	0.02	130
5	25.1	4.1	6.1	3.6	0.02	180
Mean	23.6	4.1	5.8	3.3	0.02	165

Increases of free amino acids (7) and of aspartic and glutamic amino acids (13) in chlorotic leaves have been reported.

In both sets of leaf samples, the protein/N ratio is different from the conventional factor, 6.25. Incomplete recovery of total nitrogen in the Kjeldahl method may account for this, although salicylic acid and sodium thiosulphate were used for the reduction of nitrites and nitrates to ammonium salts. The reduction is incomplete, especially when the sample is wet

TABLE II

PROTEIN IN CORN LEAF FRACTIONS

REPLICATIONS	1	2	3	4	5	6	Avg.
Fractions			*% on dry wt*				
			Green leaves				
F_1	2.54	2.92	2.97	2.86	2.75	2.86	2.82
F_2	0.86	0.92	0.74	0.69	0.51	0.77	0.75
F_3	0.83	0.86	0.87	0.51	0.34	0.62	0.62
F_4	0.82	0.86	0.37	0.67	0.63	0.69	0.66
F_5	0.38	0.37	0.17	0.15	0.11	0.30	0.25
F_6	3.95	3.55	3.46	3.77	3.03	3.46	3.37
Total	9.38	9.48	8.38	7.74	7.37	8.70	8.47
			Chlorotic leaves				
F_1	0.57	0.54	0.49	0.37	0.49	0.54	0.50
F_2	0.63	0.74	0.54	0.48	0.57	0.54	0.56
F_3	0.51	0.51	0.57	0.66	0.54	0.49	0.55
F_4	0.57	0.54	0.31	0.37	0.43	0.48	0.45
F_5	0.09	0.23	0.20	0.23	0.23	0.23	0.20
F_6	3.40	3.43	2.95	3.11	3.20	3.29	3.23
Total	5.77	5.82	5.06	4.90	5.46	5.57	5.49

(1). In the present experiment green samples containing more than 80 % moisture were used directly for nitrogen estimation. Published values for the nitrogen contents of purified proteins range from 14 to 19 %, and use of the traditional factor, 6.25, depends upon the assumption that all proteins contain 16 % nitrogen. In chlorotic leaves the low protein/N ratio is due to the presence of non-protein nitrogen, as already explained above.

Protein analysis of various fractions (table II) revealed F_6 contained the greatest amount, with a gradual decrease in protein from F_1 to F_5. The pattern of leaf protein distribution was not the same in plants from both treatments. It is interesting that while there is an appreciable difference in protein contents of the corresponding fractions of the two kinds of leaves from F_1 to F_5, very little difference is noticed in the soluble fraction, i.e. in F_6. While the fractions from F_1 to F_5 were sediments of solid particles, the F_6 fraction represented the soluble proteins of the leaf. These are considered as cytoplasmic proteins. The lower protein/N ratio in chlorotic leaves was attributed to the proportionately large amounts of soluble nitrogenous compounds not utilized in the synthesis of conjugated proteins.

Gradual decrease in protein content in the fractions of green leaves is mainly due to the decrease in the bulk of the sediment obtained at different centrifugal forces. From this data it is not possible to infer the actual protein content of particles representing these fractions. The data in table II simply show relative distribution of these fractions in the whole leaf.

SUMMARY

The effect of iron chlorosis on the pattern of protein distribution in corn leaf tissue was studied. Corn plants were raised under controlled conditions in solution culture, and leaf samples were collected from green and chlorotic plants. Leaf homogenates prepared from the samples were subjected to fractionation by centrifugation at varying speeds and the fractions were separated and analyzed for their protein and nitrogen contents.

There was no appreciable difference in total nitrogen from green and chlorotic leaves. A 25 % reduction was found in the protein content of chlorotic leaves, which gave a reduction in the protein to nitrogen ratio.

The fractions were examined under a phase microscope with chloroplasts found mainly in the first fraction, nuclei and starch grains in the second and third fraction, mitochondria in the fourth fraction, and possibly microsomes in the last fraction sedimented.

Iron chlorosis caused an 82 % reduction in the protein content of the chloroplastic fraction of the leaf. The quantity of proteins of the supernatant from the last fraction sedimented (cytoplasmic proteins) was apparently not changed by chlorosis.

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GERMINATION INHIBITORS RELATED TO DORMANCY IN WHEAT SEEDS[1,2]

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Dormancy of seeds at maturity and for an extended period of time following harvest is of great importance to the life cycle of plants. Of the many investigations of this subject, those few which pertain to wheat indicate that varieties with red kernels are more resistant to sprouting than those with white kernels (1, 5, 6, 7, 10, 12). All wheat varieties with white kernel color readily sprout at maturity (3); no exception has been found to date. In the varieties with red kernel color, varying degrees of dormancy ranging from strong to no dormancy at maturity and in the post harvest period have been observed. We initiated this study to determine the nature of dormancy in wheat and the possibility of breeding white wheat varieties with some of the dormancy characteristics of the red wheats.

Many investigators have suggested numerous causes for dormancy in wheat. We have reinvestigated the following main hypotheses: A: impermeability of the seed coat to oxygen, B: impermeability of the seed coat to water, C: immature or dormant embryo, D: mechanically tough seed coat, and E: the presence of germination inhibitors in the seed coat. Some of these hypotheses arise from observations of morphological differences between kernels of wheat with red and those with white seed coats (2, 6). The seed coat of the red wheats tightly covers the embryo whereas the seed coat of the white wheats is often separated from the embryo. This difference suggests that water might enter the embryo of the white wheat more easily than that of the red wheat varieties. Wellington (12) reported that the behavior of the covering or bran layers during germination indicated a difference in the mechanical properties of these layers in red and in white wheat kernels.

After presenting data to disprove the first four hypotheses for wheat dormancy, we report data on the presence and partial purification of some of the germination inhibitors in the seed coat of wheat. Mosheov (9) first demonstrated the presence of germination inhibitors in wheat kernels. Miyamoto and Everson (8) investigating dormancy in wheat found a parallelism between the degree of pigmentation and the amount of catechin and catechin-tannins in the seed coat during the late dough stage of maturity. They suggest that the precursors of the seed coat pigments inhibited the germination of the embryo.

MATERIALS & METHODS

Names, seed coat color and dormancy at maturity of the varieties of *Triticum vulgare L.* used in this study, are included in table I. The newly harvested red-seeded varieties which did not germinate on moistened filter paper after at least 8 days were classed as

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dormant. Those varieties which had germinated by 3 days under these conditions were classed as non-dormant.

For manometric measurements of oxygen uptake. 20 wheat kernels were incubated with 1.5 ml of water in a 15 ml Warburg vessel at 30 C. To test the rate of water uptake of wheat embryos, we placed 30 kernels on moistened filter paper and at specified times removed the embryos with a needle and measured their wet weight. We then air dried the embryos for 7 days, reweighed, and calculated the water which had been present as a percentage of the dry weight. To study embryo maturity and dormancy, the seeds were harvested in the field at 5 day intervals from 5 to 30 days after anthesis. These seeds were air dried, stored for 3 weeks and then samples of 10 seeds weighed and germinated.

A procedure which utilized hydrochloric acid and formaldehyde was applied for determining catechin plus catechin-tannin content (8).

For large scale isolation studies of inhibitory substances, 100 pounds of two wheat varieties, Red Coat and Genesee, were harvested when the moisture content was 16 %. The bran from these samples was removed within 3 days with a Buhler mill at the United States Department of Agriculture, Soft Wheat Quality Laboratory, Wooster, Ohio. The bran was placed in plastic bags and refrigerated with dry ice during shipment to the laboratory. The samples were stored at −18 C to retard dissipation of the inhibitory compounds until fractionation procedures could be initiated. For earlier and exploratory isolation studies, freshly harvested whole grain was used from both field-grown and greenhouse-grown wheat.

Bioassay: In exploratory experiments, inhibition of germination of the intact, non-dormant, white wheat seed with extracts from a dormant red wheat was found to be a very insensitive assay. Germination of excised wheat embryos on the other hand was readily inhibited by extracts from dormant wheat seeds. Therefore a wheat embryo bioassay test of inhibitor potency was developed which utilized embryos from old Red Coat wheat which had lost all dormancy. The embryos were separated from the endosperm with a razor blade. One side of the embryo was covered with the seed coat and pericarp; the other side contained a small amount of adhering endosperm. In germination tests 13 embryos were placed with the endosperm side down on a double thickness of 5.5 cm Whatman 1 filter paper discs which had been soaked with 1.5 ml of test solution in a petri dish. The dish was then incubated at 18 C. In the absence of any inhibitor in the test solution the embryos germinated uniformly after 15 hours. This procedure will be referred to as the wheat embryo bioassay. One unit of inhibitor was defined as that amount of material in aqueous solution which prevented germination for 24 hours under these conditions. Experimentally it was necessary to test a serial dilution of a solution to determine the maximum number of inhibitor units present.

A lettuce seed bioassay was sometimes utilized during the latter part of the investigation. In this assay 25 Grand Rapid lettuce seeds were germinated under conditions similar to those for the wheat embryo bioassay except that the petri dishes were incubated in the laboratory at room temperature and light intensity. Complete germination of the lettuce seed occurred overnight in the absence of inhibitory substances.

Results & Discussion

Respiration of Whole Grain: The oxygen uptake of 20 kernels of wheat from the beginning of water imbibition was measured for each of four varieties which differed in their dormancy (fig 1). These seeds were harvested in a late-dough stage and tested within a few days. Since the seeds were not sterilized, microbial respiration may have accounted for part of the oxygen uptake. Nevertheless, there was no significant varietal difference in oxygen uptake by the different seeds during the 10-hour duration of the experiment. This initial stage of oxygen uptake represents the period of oxygen permeability into the seed; in this time the non-dormant wheat varieties, Yorkwin and Genesee, had not sprouted. The data indicate that there was no difference between dormant and non-dormant seeds in the initial respiration rate and permeability of the seed coats to oxygen. The increasing rate of oxygen uptake with time of water imbibition agrees with previous studies on respiration during early stages of Big Boston lettuce seed germination (4).

Water Absorption by Whole Grain & by Embryos: The results of water-uptake studies by 2-

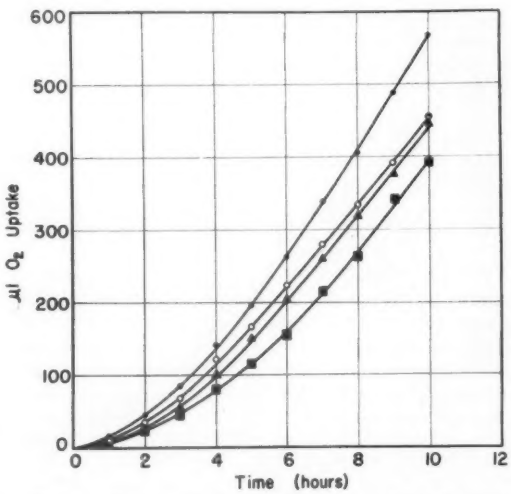

Fig. 1. Oxygen uptake by dormant and nondormant wheat seeds. Varieties were the nondormant white seeds of Yorkwin (●——●) and Genesee (▲——▲) and the dormant red seeds of Skandia (■——■) and Turkey (○——○).

TABLE I

WATER ABSORPTION BY WHEAT SEEDS AT 30 C

VARIETY	CI No.*	COLOR	DORMANCY AT HARVEST	TIME (hr) 0	4	12	24	82
				g WET WT				
Cornell 595	CI 12372	White	Nondormant	2.0	2.6	3.0	3.2	3.5
Genesee	CI 12653	"	"	2.0	2.6	3.0	3.2	3.6
Yorkwin	CI 11855	"	"	2.0	2.6	3.0	3.2	3.4
Knox	CI 12798	Red	"	2.0	2.6	3.0	3.1	3.3
Thorne	CI 11856	"	Intermediate	2.0	2.6	3.0	3.2	3.5
Skandia		"	Dormant	2.0	2.4	2.9	3.0	3.3
Red Coat	CI 13170	"	"	2.0	2.6	3.1	3.2	3.5
Turkey	CI 1558	"	"	2.0	2.6	3.0	3.2	3.5
Yga Blondeau		"	"	2.0	2.5	3.0	3.2	3.4

* CI refers to the cereal investigation collection number of the USDA.

gram samples of each wheat variety (table I) indicate no varietal difference over a period of 48 hours. Therefore, it is probable that the great differences in germination rate of these wheats cannot be attributed to a difference in permeability of the seed coat to water.

The rate of water uptake by the embryos from grains that had imbibed water for 6, 12, and 24 hours is given in table II. The embryos from all the varieties showed an increase in their water content concomitant with water uptake by the whole seed (table I). Varietal differences in dormancy could not be attributed to the small differences in rate of water intake by the embryos.

TABLE II

WATER ABSORPTION BY WHEAT EMBRYO*

VARIETY	TIME (hr) 6	12	24
	% WATER CONTENT		
Cornell 595	58	88	100
Genesee	35	70	87
Yorkwin	30	55	91
Knox	40	52	126
Thorne	57	75	100
Scandia	24	56	136
Red Coat	59	82	82
Turkey	10	54	135
Yga Blondeau	...	55	84

* Water absorbed is expressed as a percentage of the air-dry weight.

EMBRYO MATURITY & DORMANCY: The possibility of embryo maturity as a cause of dormancy was investigated in two ways. Seeds from 5 to 30 days after anthesis were harvested, dried immediately to 12 % moisture, and stored for 3 weeks. Measurement of their dry weight indicated the size of these seeds (table III). Percentage germination of the whole seeds was measured in the usual way on moistened filter paper. Seeds which had been only 5 days of age at harvest germinated 20 to 30 %, whereas all seeds 10 days or older germinated. It thus appears that the embryos in relatively immature small seeds are capable of germinating.

Further tests concerning embryo dormancy were run on the excised and isolated embryos, whose preparation was described in the methods section. Isolated embryos from Red Coat or Turkey, which were red, dormant wheat varieties, and from the non-dormant white wheat varieties, Genesee, Cornell 595, and Yorkwin, all showed greater than 80 % germination within 15 to 20 hours at 18 C. There was no significant difference in germination rate of the isolated embryos from dormant or non-dormant wheat providing the filter pads were of sufficient size to permit leaching of inhibitors from the embryos as discussed in a subsequent section. Thus embryo immaturity would not seem to be important factors in post-harvest dormancy of certain varieties of wheat.

TABLE III

GERMINATION OF IMMATURE WHEAT SEED

VARIETY	TIME OF HARVEST AFTER ANTHESIS, days	WT OF 10 DRY SEEDS, g	GERMINATION* %
Genesee (white wheat)	5	0.15	20
	10	0.55	90
	15	1.20	100
	20	2.45	100
	25	2.90	100
	30	4.30	100
Turkey (red wheat)	5	0.15	30
	10	0.60	100
	15	0.95	100
	20	2.20	100
	25	3.20	100
	30	3.55	100

* Seeds dried to 12 % moisture and stored 3 weeks before germination tests.

MECHANICALLY TOUGH SEED COAT & DORMANCY: Our observations on the germination of excised embryos indicated that a mechanically tough seed coat was not a major factor of post-maturity dormancy. In excised embryos the bran or seed coat layer from red wheat tightly adheres to the embryo surface. Should the seed coat be tougher on dormant varieties, one would expect during germination to see the seed coat separate at the periphery of the germ where it had been cut free from the endosperm. The sprout would then emerge from under the bran flap. This was not the case. During the rapid germination of the isolated embryos from dormant whole grains, normal longitudinal breaks occurred in a central position on the piece of seed coat covering the embryo. The peculiar breaks in the bran coat over the embryo of whole grains of red wheat varieties during germination were discussed by Wellington (12). Our failure to observe this consistently or to observe it with isolated embryos does not support the theory of a mechanically tough seed coat as a cause of post-maturity dormancy.

LEACHING OF INHIBITORS FROM ISOLATED EMBRYOS: In other studies concerning germination of seeds on filter paper, the rate of germination increased with an increasing ratio of the size of filter paper or volume of water per seed (11). This phenomenon has been attributed to the leaching of inhibitor substances from the seed. Similar experiments were run with wheat seeds (table IV). Thirteen excised embryos from freshly harvested and dormant Red Coat wheat were placed on water-saturated filter papers of different sizes which held correspondingly different quantities of water. More rapid germination of embryos and growth of the roots and coleoptile occurred on the larger sized filter papers. From the data was suggested the presence of inhibitors of germination and of growth which were being leached from the adhering endosperm or seed coat around the excised embryos. It was not possible to test the embryo itself for inhibitory substances. The inhibition of root or coleoptile length was a more sensitive bioassay than germination, but it was not utilized because of the necessity of making many small measurements.

LOCALIZATION OF INHIBITORS: Water extracts were prepared from about one gram of air-dried bran (seed coat, pericarp, & aleuron) and from about three grams of air-dried endosperm from 185 greenhouse-grown seeds which were harvested at the hard-dough stage. Each sample was ground and extracted with 15 ml of water at room temperature for 3 hours. These extracts were then tested in a qualitative manner by the wheat embryo bioassay. No inhibition of germination was observed with extracts of the endosperm. However, pronounced inhibitory effects were exhibited by extracts from the bran (table V). The water extracts of the bran from the red, dormant varieties, Turkey and Red Coat, were strongly inhibitory. The extracts from the white, non-dormant varieties, Genesee and Federation, were considerably less inhibitory.

These results from the water extraction of the bran, when considered with the experiments on the leaching of inhibitory substances into different sizes of moistened germination pads, indicated that an important cause of wheat seed dormancy was the presence of water soluble inhibitory substances which were located in the seed coat and not in the endosperm.

ISOLATION & SEPARATION OF INHIBITORS INTO CHLOROFORM-SOLUBLE & CHLOROFORM-INSOLUBLE FRACTIONS: The inhibitors were fractionated (fig 2). In early experiments a 100 g sample of Turkey

TABLE V

GERMINATION INHIBITORS IN WHEAT BRAN*

VARIETY	CONC. OF EXTRACT**				
	¼	½	1	2	4
Genesee (nondormant)	O	O	O	S	I
Federation (nondormant)	O	O	O	S	I
Red Coat (dormant)	O	S	I	I	I
Turkey (dormant)	S	I	I	I	I

* I = extracts were inhibitory in the wheat embryo bioassay; S = slightly inhibitory; O = non-inhibitory.

** Extracts were the supernatnt fluid prepared by soaking the bran from 185 seeds with 15 ml of water for 3 hours. Starting with this concentration, the solution was also assayed after diluting one-half and one-fourth or concentrating two- or fourfold.

TABLE IV

GERMINATION OF ISOLATED EMBRYOS FROM DORMANT RED COAT VARIETY OF WHEAT ON DIFFERENT SIZES OF WATER SATURATED FILTER PAPER*

DIAM. OF FILTER PAPER mm	NO. OF GERMINATED EMBRYOS		LENGTH OF COLEOPTILE		LENGTH OF PRIMARY ROOT		LENGTH OF SECONDARY ROOTS	
	24 hr	48 hr	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr
			mm	mm	mm	mm	mm	mm
5	8	13	1.5	2.0	0.8	5.8	1.0	3.0
7	9	13	2.2	4.1	1.9	8.1	2.9	6.8
9	10	13	4.6	6.2	5.0	11.0	3.9	9.5
14	12	13	6.0	8.0	8.8	12.3	4.2	10.5

* Conditions of the experiment were those of the wheat embryo bioassay, except for the size of the paper pad.

wheat seed was harvested in the late-dough stage, ground, and extracted with water. This wheat at this stage of development should have contained nearly maximum amounts of inhibitors. The total units of inhibitory activity for each step in the procedure were determined by the assay with wheat embryos. Later experiments were preferably started with wheat bran. The initial extraction should be done with large volumes of water. When 1 g of bran per 5 ml of water was used, a second water extraction would remove more inhibitory material. Water extraction was much more effective at room temperature than at 100 C. Boiling the bran with water for 10 minutes, at the beginning of the extraction, reduced by nearly 50 % the amount of inhibitory substances which could be obtained. This could have been due to heat lability (as discussed later), to inactivating an enzymatic release process, or to binding the inhibitors in the thick dough which was formed.

After water extraction, the debris was removed

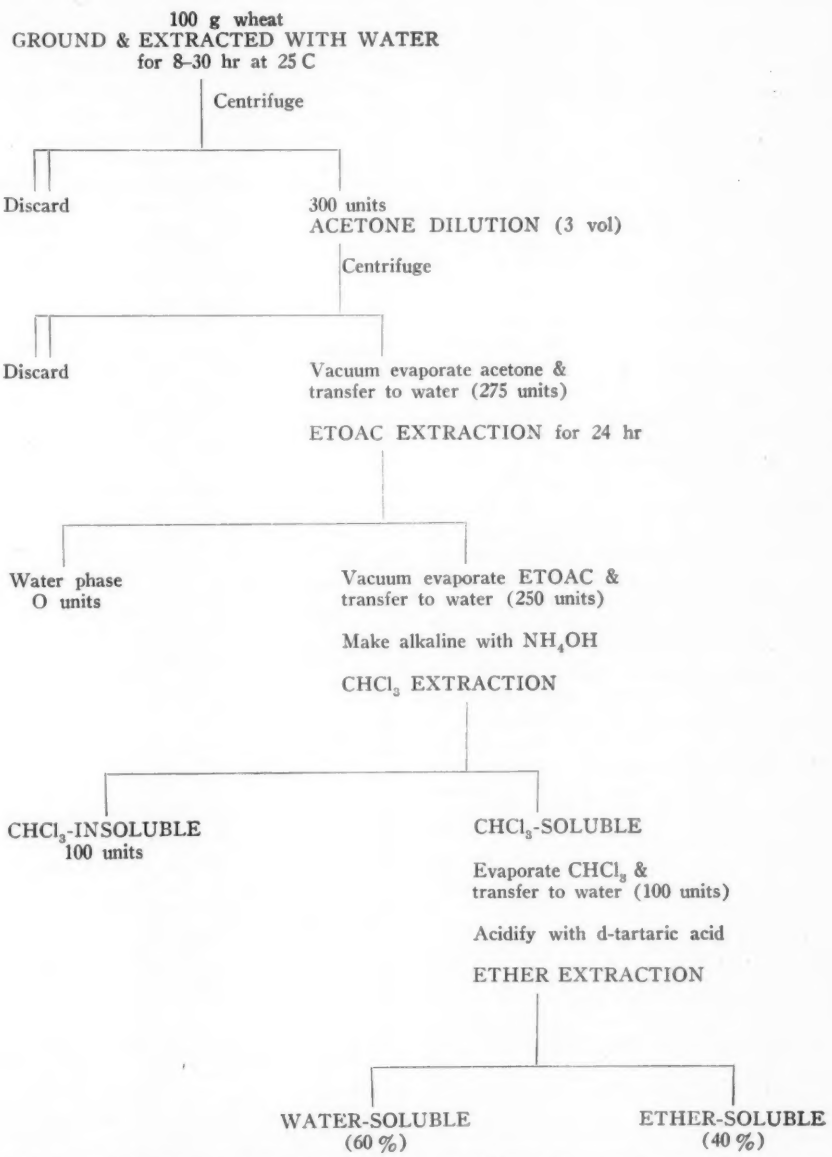

FIG. 2. Fractionation procedure for germination inhibitors in wheat bran.

by centrifugation or filtration and discarded. In order to coagulate the extracted water soluble protein, acetone was added to the supernatant and the precipitate removed and discarded. Combining these first two steps into one procedure by extracting first with water and then diluting with acetone before centrifuging off the residues resulted in the recovery of an equal amount of activity. However, the acetone also extracted fats and lipids from the insoluble residues, and these fatty constituents interfered in subsequent purification steps. Therefore we recommend a separation of the residue from the initial water extract before adding acetone in order to obtain purer preparations.

The inhibitory substances were removed by a continuous ethyl acetate extractor for 24 hours. After evaporation of the ethyl acetate and transfer of the inhibitors to an aqueous solution, NH_4OH was added until the solution was alkaline to litmus paper; then it was extracted with chloroform. About half the inhibitory activity was soluble in chloroform and was designated as chloroform-soluble. Half the activity remained in the water phase and was designated as the chloroform-insoluble fraction.

Inhibitors in Chloroform-Insoluble Fractions: The yellow colored chloroform-insoluble fraction contained the catechin-tannins. About 16 mg of dry weight of this crude catechin-tannin fraction per 100 ml were inhibitory in the wheat embryo assay. Fractions of this component have been obtained which were inhibitory at 10 mg per 100 ml.

The stability of the inhibitors in the chloroform-insoluble fractions was measured as a function of time at different temperatures (fig 3). Aliquots from 100 ml of an aqueous solution which contained 4,000 units of chloroform-insoluble inhibitors were treated at the designated temperatures for 24 hours. The loss of activity increased rapidly with increasing temperature. A residual activity of 25 % of the total in this fraction was heat stable. At 2 C the activity in the chloroform-insoluble fraction dropped only 13 % in 1 month. Concurrent with the loss of activity an insoluble precipitate was formed in the aqueous solution. The data suggest that the chloroform-insoluble fraction contained at least two components; one which was heat labile and one which was stable at 100 C. Another interpretation of these data would be that the active catechin-tannin component was polymerized by heat to a new structure with only 25 % of the activity of the original material.

Inhibitors in Chloroform-Soluble Fraction: The chloroform-soluble fraction, containing about half the total activity, was further fractionated (fig 2). The essential step involved adding d-tartaric acid until the aqueous solution was weakly acidic (pH 2.5) and then extracting with ethyl ether. The ether soluble fraction contained about 40 % of the activity and the fraction remaining in the water phase had about 60 % of the activity. There was little loss of the activity which had been recovered in the chloroform-soluble fraction.

From isolation experiments which had begun with 6 kg of bran from Red Coat wheat, both the fractions from the ether extraction at the end of fig 2 were investigated further. The water-soluble fraction was characterized by being soluble in ether from a weakly alkaline (KOH or NH_4OH) aqueous solution, but non-extractable by ether from an acidic aqueous solution. These are the properties of alkaloid-like materials without strong phenolic properties. The inhibitory effect of this material on seed germination was very heat labile. Boiling an aqueous solution of it for 5 minutes produced a completely inactive material when tested by the bioassay. The material was transferred between water and ether two times. The active material was then transferred to ether from an alkaline ($NaHCO_3$) aqueous solution, the ether was removed by evaporation and a white amorphous material was obtained which retained most of the biological activity.

The other fraction of inhibitors was characterized by being chloroform-soluble from an alkaline aqueous solution and ether soluble from an acidic aqueous solution (fig 2). Most of this material was soluble in hot water, but only slightly soluble in cold water. By repeated crystallization from hot water, it was possible to purify a substance in this fraction to a constant melting point of 102.5 C. However, the material was not very stable in hot water, and it was necessary to discard a biologically inactive, hot water insoluble, decomposition product after each recrystallization. The crystalline material (50 ppm) inhib-

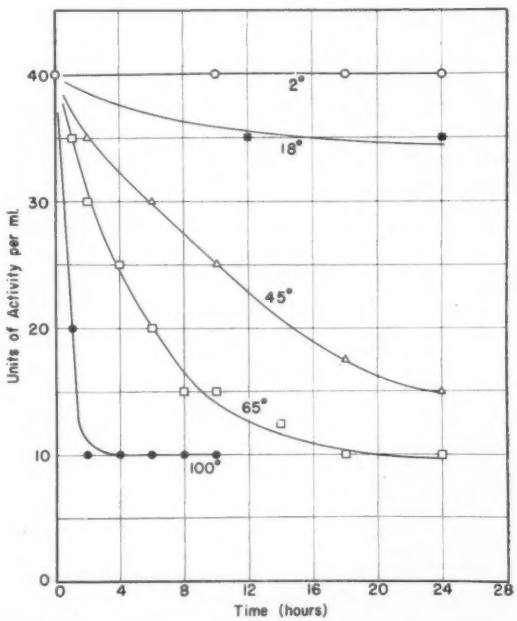

Fig. 3. Time-temperature relationships for the loss of inhibitory activity of the catechin-tannin (chloroform-insoluble) fraction from the bran of the dormant Red Coat wheat.

ited the germination of excised wheat embryos for 24 hours. The germination of whole lettuce seed in the light was not carefully determined because of lack of material, but 5 to 50 ppm of this unknown completely inhibited lettuce seed germination and 5 ppm or less slowed down the germination rate. We shall investigate further the characterization of the material.

NUMBER OF INHIBITORS IN WHEAT BRAN: From this work it appears that the seed coat of wheat contains numerous substances which inhibit the germination of the wheat embryo. Isolation in a pure form and identification of these components will require much further work. The inhibitory substances in this study were all extracted from the seed coat because of their solubility in water at room temperature. They were not extractable from the bran by acetone, ether, or 95 % ethanol, although they were soluble in these solvents after extraction by water. This inconsistency in solubility might reflect some permeability characteristic of the seed coat.

The isolation procedure used for the inhibitory subtances indicated that there were four different types of inhibitors present. A: About 38 % of the total inhibitory activity was in a chloroform-insoluble fraction and was heat-labile. B: About 12 % of the total inhibitory activity was also in the chloroform-insoluble fraction, but was heat stable. The latter was either a second compound in the chloroform-insoluble fraction or represented the conversion of the heat-labile component to a heat-stable derivative. According to the isolation procedure these two types of substances were catechin- and tannin-like materials. C: About 30 % of the total inhibitory activity behaved as if it were alkaloid-like, since it was more soluble in chloroform than in an alkaline aqueous solution, and more soluble in acidic water than in ether (4). From the ether-soluble fraction a fourth component, representing about 20 % of the original activity, was obtained as an unknown crystalline material. How many of these inhibitory compounds in the wheat seed coat may actually be involved in dormancy is not known.

The number of inhibitory substances detected in wheat suggests the reason why plant breeding research for dormant white wheat has been unsuccessful. If there are many inhibitors for dormancy, the plant breeder will have to locate genetic markers or analytical procedures for detecting each before a dormant white wheat can be obtained. Further, each inhibitor should not be considered alone, but rather as a member of a group of inhibitors. These inhibitors may interact and reinforce dormancy depending on threshold values of each which may be reached over various periods of time.

STABILITY OF INHIBITORS IN VIVO: Dormancy in seeds is not a permanent phenomenon, but one which disappears slowly with time. Wheat seed which is dormant at harvest generally will germinate 3 weeks after harvest. If inhibitors control dormancy, then there must be a loss or change in the inhibitory substances in the post-harvest period which coincides with the loss of dormancy. In the previous section we demonstrated that most of the inhibitory substances in the chloroform-insoluble material were heat labile in their isolated form (fig 2). Also both components of the chloroform-soluble fraction, the alkaloid-like material, and the crystalline unknown, were very labile at 100 C. Consequently the slow loss of these materials with time in vivo at room temperature would explain the loss of dormancy. This postulate was investigated in two experiments. In the first a direct determination was made of the catechin-tannin content of seed coat material which had been collected from wheat in the late-dough stage or 3 weeks after harvest (table VI). This assay was selected because a major fraction of the inhibitory activity which had been isolated was in the catechin-tannin fraction. In the three varieties of wheat there was a loss within 3 weeks after harvest of at least 67 % of the catechin-tannin content that had been present at the time of harvest. At harvest and 3 weeks after harvest the catechin-tannin content of the two dormant red wheat varieties, Red Coat and Turkey, was twice as high as that of the non-dormant white wheat variety, Genesee.

TABLE VI

CATECHIN PLUS CATECHIN-TANNIN CONTENT OF SEED COAT OF WHEAT

VARIETY	TIME OF ANALYSIS	
	LATE-DOUGH STAGE mg %	3 wk AFTER HARVEST mg %
Genesee	205	75
Red Coat	554	175
Turkey	588	131

In another experiment, designed to study the loss of inhibitors during aging of the newly harvested grain, the units of inhibitor activity were isolated by the fractionation procedure from newly harvested wheat and from wheat which had aged for 3 weeks at room temperatures (table VII). The amount of inhibitors which could be isolated in either the chloroform-insoluble or chloroform-soluble fraction from each of the different varieties was much less after 3 weeks of storage.

The data from these two experiments show that there is a great loss in the amount of inhibitors during aging in the immediate post-harvest period. The amount of this loss was also dependent upon the variety of the wheat. When the amount of these inhibitors has decreased to a certain level, germination should then be possible. It is assumed that the level of these inhibitors in the non-dormant white wheat remained below a threshold amount which inhibited dormancy. The level of the catechin-tannin content of seed coats of red wheat had dropped in 3 weeks to a value equal to or less than the catechin-tannin content in newly harvested white wheat (table VI) and both types of wheat germinated. Likewise the level of inhibitors in both the chloroform-insoluble and chloroform-soluble fractions from red wheat decreas-

TABLE VII

EFFECT OF GRAIN AGE UPON UNITS OF INHIBITOR WHICH COULD BE EXTRACTED

VARIETY	LATE-DOUGH STAGE		3 wk AFTER HARVEST	
	$CHCl_3$-insol.	$CHCl_3$-sol.	$CHCl_3$-insol.	$CHCl_3$-sol.
Genesee	37	6	21	Trace
Yorkwin	32	13	22	8
Cornell 595	39	12	14	5
Red Coat	50	44	21	6
Turkey	110	90	26	15

ed in 3 weeks to a value equal to or less than the amounts of these substances present in the white wheat at harvest (table VII).

REVERSAL OF INHIBITION BY GIBBERELLIN: The stimulative effect of gibberellin on seed germination is well known. Simultaneous application of inhibitors of germination and of gibberellin may indicate interrelationships in the action of these compounds. The dormancy of wheat embryos induced by one unit of the catechin-tannin fraction was reversed in part by about 10 ppm of gibberellin A_3. The effect of one unit of the crystalline unknown inhibitor from the chloroform-soluble, ether-soluble fraction on wheat embryo germination was completely reversed by 10 ppm gibberellin A_3. Inhibition of wheat embryo germination by 20 units of the inhibitors was so effective that 50 ppm of gibberellin A_3 did not reverse the inhibition. When lettuce seed germination was tested, one unit (as measured by the wheat embryo test) of either the catechin-tannin material or of the crystalline unknown produced a more prolonged inhibition of germination of lettuce seeds. This inhibition was not reversed by 50 ppm of the gibberellin, nor did the presence of gibberellin A_3 increase the length of the roots when germination did occur. Thus the inhibition of lettuce seed germination in the light appeared to be a more sensitive assay for the inhibitors than was that with wheat embryo, but it was not reversed by gibberellin A_3. Lettuce seed germination could be used for a bioassay in future work providing that all the inhibitors affected both lettuce seed and wheat embryos in a similar manner.

SUMMARY

A wheat embryo bioassay was used to detect the presence of naturally-occurring inhibitors which affect the dormancy of wheat seed. Post-harvest dormancy in wheat was caused by inhibitors which were located in the seed coat. Dormancy was not caused by restricted water or oxygen uptake, a mechanically tough seed coat, or immature embryos. The loss of dormancy during the weeks after maturity was correlated with the natural inactivation of the inhibitors. At least four inhibitory fractions were extracted from the wheat seed coat. A: A water-soluble, chloroform-insoluble fraction, designated as a catechin-tannin fraction, was heat labile. B: A part of the activity of the catechin-tannin fraction was heat stable and represented either a second component or a biologically active decomposition product of the unstable material. C: From the chloroform-soluble fraction, a component was isolated which was more soluble in acidified water than in ether and was considered to be an alkaloid. D: An unknown inhibitory compound was isolated and crystallized from the chloroform-soluble material on the basis of its solubility in ether and insolubility in cold water. It represented about 20% of the total inhibitory activity of the wheat bran. About 50 ppm of this material inhibited the germination of wheat embryos, and it was about ten times more effective in depressing lettuce seed germination. Gibberellin A_3 could reverse the inhibitory effect of this unknown on wheat embryo germination if the inhibitor was not present in excess. The inhibitory effect on lettuce seed germination was not reversed by gibberellin A_3.

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IDENTIFICATION & MODE OF ACTION OF A COMPONENT OF POSITIVELY-IONIZED AIR CAUSING ENHANCED GROWTH IN PLANTS [1, 2]

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INTRODUCTION

Ever since Maimbray in 1746 electrified two myrtle bushes and reported "they put forth small branches and blossomed sooner than other shrubs which had not been electrified" (15), investigators have been subjecting plants to all manner of electrical shocks. Typical experiments are described by J. C. Bose (4) and K. Stern (19). When E. C. Barton-Wright surveyed the entire field in 1933, he concluded that in general the results had been "remarkable poor" (1).

No doubt many of these experiments, especially those employing high voltage discharges (3), resulted in variable numbers and kinds of air ions being present in the vicinity of the plants. But the extent to which this may have influenced the results is now impossible to determine.

During the last 6 years, the effects of air ions per se on mammalian cells and enzyme systems have been investigated in detail (8, 9, 10, 11, 12). A mechanism of action has been hypothesized (13) and has received partial experimental confirmation (14).

In order to investigate the action of gaseous ions on plants, and in the absence of any comparable work, we decided to begin this study with some relatively simple photosynthsizing organism. After screening many species of green and blue-green algae (including *Chlamydomonas reinhardi, Protococcus viridis, Chlorella pyrenoidosa, Nostoc muscorum, Scytonema hofmanni,* & *Anabaena spiroides*) we found that the common blue-green alga, *Microcoleus vaginatus,* displays an easily detectable response to positively-ionized air.

Although we later found that positively-ionized air produces an analogous response in other algal species and in higher plants, considerably more elaborate techniques are required for its demonstration. The remarkable hardiness of Microcoleus and its capacity for rapid, aggressive growth under favorable conditions make it ideal for experiments of this type.

MATERIALS & METHODS

A. IDENTIFICATION OF A GROWTH-STIMULATING COMPONENT OF POSITIVELY-IONIZED AIR. A specimen of *Microcoleus vaginatus* (Vaucher) Gomont was isolated from a local soil crust. Stock cultures of this strain were maintained on the Medium I of Sager and Granick (17). Although this medium was developed for *Chlamydomonas reinhardi,* it proved upon addition of 2 % agar to be especially favorable for Microcoleus growth. Cultures were incubated at room temperature in water-saturated atmospheres and were exposed to 1,000 ft-c for 12 hours each day.

The ion source was a Wesix "Ionaire" consisting of tritium in a sealed foil with a reversible rectifying circuit that permitted selection of either positive or negative air ions (2). Unipolar ion densities of $1 \times 10^8/cm^2/sec$ were produced by this apparatus, as measured with a Beckman micro-microammeter used in conjunction with the Beckett target probe. The monitoring technique and the calculations involved have already been described in detail (11).

An exposure chamber was adapted from the glass manifold of a micro-Kjeldahl apparatus. It consisted of a horizontal glass cylinder, 22 inches long and 2 inches in diameter, through which water-saturated air was continuously drawn. The tritium head of the ion source was placed inside the cylinder, just under the roof, in about the center of the chamber.

Single Microcoleus hormogones or 1.0 mm discs excised from the stock culture were placed on the center of fresh 5 ml plates of Sager and Granick agar. These plates were then arranged lengthwise in the exposure chamber. Those plates located upstream of the ion source were the control samples; those plates downstream of the ion source were the test samples. No plate was located closer than 5.0 cm to the ion source.

Distilled water that had been freshly boiled and cooled was used to saturate the air in the growth chamber. When pure gases were alternated within the chamber with air, the water was changed with each change in the ambient atmosphere. A conventional CO_2-absorption train filled with Ascarite was used to remove carbon dioxide when required.

RESULTS

EFFECT OF AIR IONS ON MICROCOLEUS GROWTH: The positively-ionized discs of *M. vaginatus* showed an increased growth rate over the controls that became readily apparent to the naked eye within 1 or 2 days. When single hormogones were exposed, it was

[1] Received May 31, 1961.

[2] A report of this work was given April 17, 1961 at the 37th regional AAAS Meeting, Tempe, Ariz.

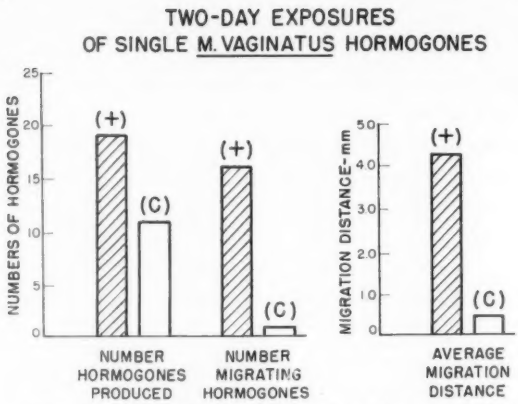

FIG. 1. Effect of positive air ions on the growth and migration of single *M. vaginatus* hormogones.

possible to put this increased growth in quantitative terms (fig 1).

Under similar conditions, negative air ions had no detectable effect on algal growth.

IDENTIFICATION OF GROWTH-STIMULATING COMPONENT: Since air is a mixture of gases, ionized air is a mixture of gaseous ions. To determine which of the gaseous ions (present in positively-ionized air) was responsible for the observed effect, we performed the experiments shown in figures 2 and 3. Arbitrary growth classifications were defined, based on the numbers and distances of the migrating filaments. Test conditions were alternated with normal growing conditions each hour, to insure continued viability of the Microcoleus.

The increased growth effect was strictly dependent on the presence of positively-ionized carbon dioxide. No other ionized gas had any detectable effect upon algal growth under these experimental conditions.

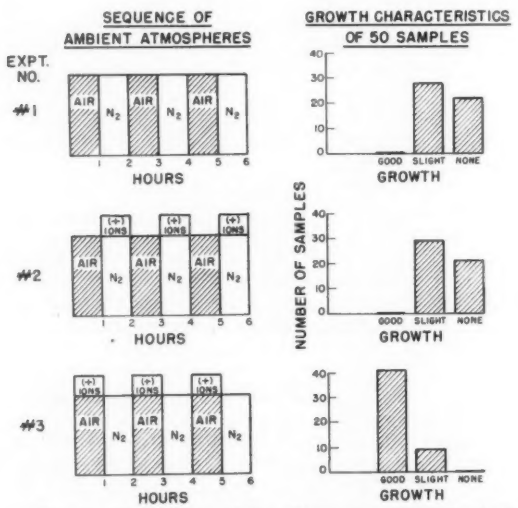

FIG. 2. Comparison of the effects of positively-ionized nitrogen and positively-ionized air on *M. vaginatus* growth.

DISCUSSION

There is no theoretical reason for supposing carbon-dioxide-minus-an-electron to be more efficient in photosynthesis than un-ionized carbon dioxide. Quite the reverse, in fact, since the transformation of carbon dioxide into sugars is a reductive process. Moreover, the rate at which illuminated spinach chloroplasts reduce 2, 6-dichlorophenol is not altered by positive air ions, so it seems unlikely that the Hill reaction is involved.

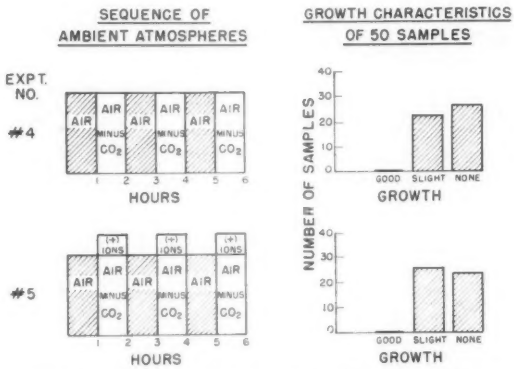

FIG. 3. Abolition of the growth-stimulating effect by the removal of carbon dioxide from positively-ionized air.

Positively-ionized carbon dioxide is also the mediator of positive air ion effects on mammalian tissue. Here it apparently acts by causing a local release of 5-hydroxytryptamine within the cell (19).

In plant cells, positively-ionized carbon dioxide might conceivably cause a local release of compounds structurally related to 5-hydroxytryptamine, such as indole-3-acetic acid. Thus, the mechanism of positive air ion action on plants and animals would be essentially the same. The different responses (enhanced growth in plants & vasoconstriction in animals) would be due simply to the different properties of free indole-3-acetic acid and free 5-hydroxytryptamine within their respective tissues.

With a view toward testing this hypothesis, the following experiments were undertaken.

MATERIALS & METHODS

B. RELEASE OF BOUND INDOLEACETIC ACID AS MECHANISM OF ION-INDUCED GROWTH STIMULATION. The same stock culture of *Microcoleus vaginatus* and the same growth medium described in part A were

used here. 5 mm discs were excised from the stock cultures with a cork borer and placed on the surface of conventional petri dishes containing 20 cc of the agar medium. Concentrations of indoleacetic acid (Fisher) ranging from 0.01 to 200.0 μg per plate were either incorporated directly into the medium, or placed on filter paper discs (Difco sterile blanks, 6.5 mm) on the agar surface and allowed to diffuse into the medium.

Experiments on oat and corn were performed in light-proof growth chambers consisting of enameled metal bowls, 7.0 cm high and 15.0 cm in diameter, covered with canopies of opaque polyethylene film (containing 25 % carbon black). The canopies were fastened tightly around the bowls by means of rubber bands. The test growth chambers were identical with the control growth chambers except for the ion source which protruded through the center of the polyethylene canopy.

We followed the Nitsch and Nitsch modification of Bentley's Avena section straight-growth test (16). The 4.0 mm oat coleoptile sections were floated in watch glasses or 5 ml beakers, which were placed inside the growth chambers. Atmospheres within both control and test growth chambers were water-saturated.

Indoleacetic acid was assayed by means of the colorimetric method of Gordon and Weber (6). Golden Victory sweet corn was the test material in the bound-IAA experiments. Kernels and coleoptiles were exposed in the same growth chambers and under the same conditions as the Avena coleoptiles.

The ion source and density were the same as described in part A. At no time were the test materials closer than 5.0 cm to the ion source.

Results

Effect of Added Indoleacetic Acid on Microcoleus Growth: In our preliminary screening experiments, we noted that positively-ionized suspensions of *Chlamydomonas reinhardi* showed no increased growth over control suspensions (as indicated by cell counts). Yet, the filtrates of the positively-ionized suspensions had a marked growth-stimulating effect on Microcoleus. This suggested that positive air ions had caused the release of an auxin-like material which, though unable to increase Chlamydomonas growth under the given experimental conditions, was able to stimulate Microcoleus growth.

The role of hormones in algal physiology is still incompletely understood, yet many workers have reported that indoleacetic acid stimulates the growth of several Chlorophyte species. These investigations have been summarized by R. W. Krauss (7).

Since our theory postulates that increased growth is due to released indoleacetic acid, it is important to be sure that indoleacetic does in fact cause increased Microcoleus growth. Accordingly, the effect of graded concentrations of indoleacetic acid from 0.1 to 200.0 μg (3×10^{-8}–3×10^{-5} M) on excised Microcoleus discs was observed.

Enhanced growth in the treated discs was clearly visible to the naked eye within 24 hours. After 3 days, the diameter of the treated discs was from 5 to 25 times greater than that of the control discs. A maximum effect was obtained with 1.0 to 2.0 μg indoleacetic acid per plate (3–6×10^{-7} M). However, concentrations as low as 0.01 μg (3×10^{-9} M) produced unequivocal (i.e. 2 ×) growth increases.

Effects of Air Ions on Avena Coleoptiles: We next tested the effects of air ions on higher plants with better established hormonal responses.

In the Avena test, positive air ions caused a slight but significant increase in coleoptile growth. Negative air ions had no significant effect (table I).

Table I

Effect of Positively- & Negatively-Ionized Air on Growth of 4.0 mm Avena Coleoptiles*

Sample statistic	Expt. No. 5		Expt. No. 6	
	(C)	(+)	(C)	(−)
X	5.40 mm	6.13 mm	5.20 mm	5.15 mm
N	10	10	10	10
S	0.40	0.60	0.44	0.61
S.E.M.	0.14	0.21	0.14	0.19

* Nitsch and Nitsch straight-growth test (16).

The degree of increased elongation in the positively-ionized coleoptiles corresponded to an amount of indoleacetic acid in the 1.0 ml suspending medium of 5×10^{-3} μg, or 5 μg/l.

Similar results were obtained in 22 experiments involving more than 500 coleoptile sections.

Effect of Positive Air Ions on Release of Bound Indoleacetic Acid: If our hypothesis is correct, it should be possible to couple pure indoleacetic acid with suitable binding materials, then demonstrate that positive air ions cause indoleacetic acid to be released in greater amounts than under control conditions. With such a technique, the indoleacetic acid may be assayed by a colorimetric method rather than by the time-consuming Avena test.

Indoleacetic acid in aqueous solution was combined with a variety of materials: Dowex 1-×8, Amberlite IR-4B, soil, carbon, and several proteins. In no case could we demonstrate that positive air ions had the slightest effect on the release of indoleacetic acid from these materials.

Better results were obtained when germinating corn kernels were soaked overnight in 1×10^{-3} M indoleacetic acid solutions, washed thoroughly, then exposed in distilled water on watch glasses to positive air ions. As shown in table II, the amount of indoleacetic acid in the distilled water increased more rapidly in the positively-ionized sample. However, in roughly one-third of these experiments there was

TABLE II

EFFECT OF POSITIVELY-IONIZED AIR ON RELEASE BY GERMINATING CORN KERNELS OF ADDED INDOLEACETIC ACID

Hr Exposed	OD_{530} of Supernatant*		mg IAA/l in Supernatant**	
	(C)	(+)	(C)	(+)
0	0.00	0.00	0.0	0.0
4	0.12	0.13	4.0	4.0
12	0.20	0.32	6.0	9.0
24	0.33	0.45	9.0	12.5

* After treatment with the Gordon-Weber colorimetric reagent.

** Read from a freshly prepared standard curve.

no significant difference between control and positively-ionized supernatants.

A more reproduceable demonstration of ion-induced release of indoleacetic acid was obtained as follows: corn seedlings were submerged in 1×10^{-3} M indoleacetic acid overnight, the first 3.0 mm of the coleoptiles removed, washed, and exposed to positive air ions while floating on 4.0 ml distilled water. At intervals, coleoptile tips were removed, placed on spotting plates, and treated with 0.5 ml of the Gordon-Weber colorimetric reagent.

Under these conditions, the positively-ionized coleoptile tips rapidly lost their ability to form a color with the Gordon-Weber reagent (table III). This effect was not obtained when the tips were suspended in 0.01 M KCN during ionization. Similarly, no binding of indoleacetic acid occurred when the seedlings were pre-treated with 0.01 M KCN.

TABLE III

EFFECT OF POSITIVELY-IONIZED AIR ON RELEASE OF ADDED INDOLEACETIC ACID BY CORN COLEOPTILES, AS SHOWN BY THEIR APPEARANCE AFTER TREATMENT WITH GORDON-WEBER COLORIMETRIC REAGENT

Hr Exposed	Color of coleoptiles			
	Untreated		KCN-Treated	
	(C)	(+)	(C)	(+)
0	Purple	Purple	Purple	Purple
2	Purple	Mottled	Purple	Purple
10	Mottled	White	Purple	Purple

DISCUSSION

Although there is no doubt that binding of indoleacetic acid to protein occurs in plants (5), the exact nature of the bond or bonds has yet to be elucidated. Siegel and Galston have shown that conditions of active aerobic respiration are necessary for binding indoleacetic acid to pea roots (18). Such conditions are also necessary for the ion-induced release of indoleacetic acid, judging by its abolition by KCN and our repeated failure to obtain it from non-living systems.

While it seems reasonable to assume that added indoleacetic acid is bound to protein in the same way (or ways) as naturally occurring indoleacetic acid, it should be emphasized that this is, in fact, an assumption. We have shown that positive air ions will release added indoleacetic acid from plant tissue. This is not quite the same thing as showing that positive air ions will release naturally-occurring bound indoleacetic acid from plant tissue. Obviously this will have to be demonstrated before our hypothesis can be accepted.

Another question must be answered: is there enough bound indoleacetic acid naturally present in the oat coleoptile sections to produce the observed growth increases?

According to Wildman and Bonner (22), the first 5.0 mm of an Avena coleoptile contains approximately 1×10^{-4} µg free indoleacetic acid. The Nitsch and Nitsch assay uses a 4.0 mm section cut 3.0 mm from the tip. Interpolating from the concentration gradient of indoleacetic acid in the oat coleoptile as shown by Thimann (20), we may estimate that at the time of cutting the 4 mm section contains 0.5×10^{-4} µg free indoleacetic acid. According to van Overbeek (21), this represents only 8 % of the bound indoleacetic acid. Because the amount of bound indoleacetic acid varies according to the type of extraction used, the age and growing conditions of the seedling, we shall use a range of 3 % to 10 % in this calculation. Thus, a single 4.0 mm Avena section may contain from 2×10^{-3} to 5×10^{-4} µg bound indoleacetic acid. Since ten sections are used in each assay sample, 0.02 to 0.005 µg bound indoleacetic acid are present in each sample.

The lower limit of sensitivity of the Avena assay is reported to be 0.005 µg (16). Thus, in the least favorable cases there is still enough bound indoleacetic acid present to cause a detectable growth response if released. In the most favorable cases only one-fourth of the bound indoleacetic acid present would have to be released in order to cause a detectable growth response.

These calculations cannot replace the direct analyses that will have to be made. But they do indicate that our hypothesis is well within the realm of possibility.

SUMMARY

Exposure of *Microcoleus vaginatus* (Vaucher) to positively-ionized air results in increased migration and growth. Negative ions are without observable effect. The increased-growth effect, dependent upon the presence of positively-ionized carbon dioxide, can be duplicated by adding minute amounts of indoleacetic acid.

Positive air ions cause increased growth of oat coleoptiles and release added indoleacetic acid from

corn coleoptiles. The hypothesis is advanced that positively-ionized air causes increased growth in plants by releasing endogenous bound indoleacetic acid.

Acknowledgment

We wish to thank Walter Fish of Arizona Nucleonics, Inc., for the ionizing and monitoring equipment used in these experiments.

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AN INHIBITOR OF INDOLEACETIC ACID OXIDASE FROM PEA TIPS[1]

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Reduction of IAA oxidase activity in etiolated pea buds by exposure to light was first reported by Tang and Bonner (9). Hillman and Galston (4) have shown that the IAA oxidase activity of the buds of etiolated pea plants is inhibited by red light (640 mμ) and that the effect is reversed by near infrared (730 mμ) light. They presented evidence that the inhibition is due to increased concentration of a dialyzable inhibitor. Our investigation of the structure of this IAA oxidase inhibitor has revealed that it is a derivative of kaempferol. Although kaempferol and related flavanoids have been found previously in many plant species the role that these compounds play in the biochemistry of the plant has remained obscure. The finding here that a derivative of kaempferol is an IAA oxidase inhibitor suggests that flavanoids may exert an effect on plant growth through their action on the IAA oxidase system.

EXPERIMENTAL

IAA OXIDASE ENZYME: Black Valentine bean roots were used as the source of the IAA oxidase (IAAO) since they proved to be essentially free from cofactors and inhibitors of the enzyme. The enzyme was prepared from 8 day old Black Valentine bean plants grown under artificial light at 2,000 ft-c, day-length 16 hours and day and night temperatures of 23 C and 18 C, respectively. The roots were rinsed with tap water and blotted with paper towels, then cut into 1-inch lengths and dropped immediately into dry ice. Frozen root sections, 25 g, were pulverized in a chilled mortar; the resulting fine powder was mixed with 150 ml of 0.02 M KH_2PO_4 buffer, pH 6.1, and stirred for 20 minutes. Centrifugation at 20,000 × *g* for 10 minutes yielded a clear supernatant which when diluted to 250 ml with buffer yielded enzyme stock solution (E) which could be stored frozen for months with little loss of activity.

SOLUTIONS: A stock solution (S) containing the substrate IAA at 1×10^{-3} M and Mn^{++} at 5×10^{-4} M was prepared by dissolving 175 mg of IAA and 98.95 mg of $MnCl_2 \cdot H_2O$ in a liter of 0.01 M KH_2PO_4 buffer, pH 6.1. A 1×10^{-3} M stock solution of 2,4-dichlorophenol (DCP), for use as cofactor in the standard assay, was prepared by dissolving 163 mg of the compound in one liter of buffer.

ASSAY: In the standard assay for inhibitor activity, the assay cups, 50 ml beakers, contained 1 ml of E, 2 ml of S, 0.5 ml of DCP, 0 to 5 ml inhibitor solution, and sufficient 0.01 M buffer to bring the total volume to 10 ml. Samples were incubated in a Dubnoff metabolic shaker at 26 C. Residual IAA was determined with Salkowski ferric chloride reagent by the method of Tang and Bonner (9).

GROWTH OF PEAS: Peas (*Pisum sativum* L. var. Alaska), obtained from W. Atlee Burpee Co., Philadelphia, Pa., were grown in vermiculite in darkness at 23 to 25 C and 50 % relative humidity. At 7 or 8 days the plants were exposed for 20 minutes to 800 kiloergs of red light from a source similar to that of Nitsch and Nitsch (6). The terminal buds were harvested 18 to 20 hours later and dropped into a beaker cooled in dry ice. The frozen tips usually were extracted immediately after harvest but occasionally were stored frozen for a week or so.

PRELIMINARY WORK: An initial experiment was performed to confirm the red light effect on IAA oxidase (IAAO) of etiolated pea buds. Homogenates of buds from dark control and red light-treated etiolated pea plants were prepared in a fashion similar to the bean root enzyme preparation except that the pea bud homogenate stock solutions represented 500 mg tissue per milliliter. With DCP as cofactor at 5×10^{-5} M, and varying enzyme concentration, the presence of inhibitor in the red light bud homogenate was very apparent. On the other hand, homogenate of dark control buds with no added cofactor, showed increasing IAA destruction with increasing enzyme concentration.

Both homogenates were dialyzed against an equal volume of water at 4 C for 72 hours. A 10 μl aliquot of each dialysate was chromatographed on paper. Each dialysate showed the presence of substances which gave a brown color with diazotized sulfanilic acid (1) and a bright yellow aluminum lake. In 20 % KCl as solvent, the R_f of the major substance in dark control dialysate was 0.28 while that of red light dialysate was 0.12. This value (0.12) for dialysate of red light treated buds compares to the R_f 0.14 obtained later for the purified inhibitor.

[1] Received June 12, 1961.

[2] Contribution No. 697.

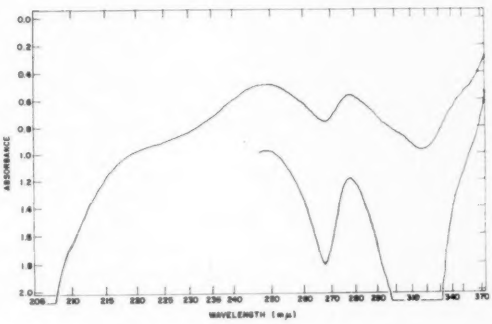
ISOLATION OF INHIBITOR: In a preliminary experiment, 200 mg of frozen red light-treated buds were pulverized in a chilled mortar and the resulting powder stirred in 25 ml of ethanol for 20 minutes, then filtered. The filtrate was evaporated to dryness and the residue was extracted with 1 ml of methanol. Removal of the methanol left a residue which was taken up in 5 ml of 0.01 M phosphate buffer, pH 6.1, and tested in the IAAO assay. At a level representing 80 mg of tissue per 10 ml reaction mixture, this solution produced 100 % inhibition of IAA destruction during 60 minutes incubation.

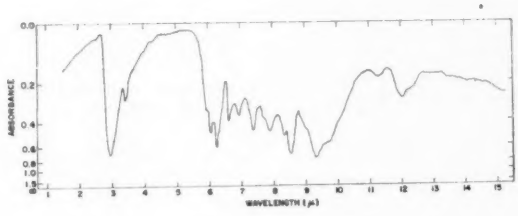
From a similar extraction a 5 μl aliquot of methanol extract was spotted on Whatman No. 1 paper and chromatographed in butanol: 27 % acetic acid (1:1). Spraying the chromatogram with diazotized sulfanilic acid (DSA) reagent (1), then 10 % sodium carbonate, revealed major amounts of two phenolic compounds with R_f's of 0.22 and 0.58. These two areas from a similar chromatogram were eluted with 0.01 M phosphate buffer. IAAO assay of these eluates indicated that the R_f 0.58 fraction was an IAAO inhibitor. On other chromatograms the R_f 0.58 spot appeared dull brown under UV light alone but fluoresced yellow-green under UV light in the presence of ammonia vapors. This spot also gave an olive color with ferric chloride and formed a yellow lake with aluminum chloride, which fluoresced yellow green under UV light. The R_f in ethyl acetate was 0.02; in 15 % acetic acid 0.68 and in 20 % KCl 0.14. These combined properties suggested that this inhibitor might be a flavonol-3-glycoside. However, flavonol glycosides normally have a UV absorption maximum in the 340 to 360 mμ region and this material exhibited maxima only at 317 and 268 mμ.

To obtain sufficient material for further study, 95.5 g of frozen red light-treated terminal buds were ground in a chilled mortar and the resulting powder was stirred 1 hour in 500 ml ethanol, then filtered. Evaporation of the filtrate yielded 4.4 g of oily yellow residue. This was chromatographed on a 1.5 × 45 cm Whatman ashless cellulose powder column using propanol: acetic acid: water (20:1:10). The eluate was cut into several 10 to 15 ml fractions and these were examined by paper chromatography. Fractions containing the inhibitor were pooled, concentrated, and chromatographed a second time on cellulose. The resulting inhibitor-containing fractions were pooled and taken to dryness yielding 1.23 g gummy yellow residue.

This residue was further purified on an LKB pressurized paper chromatographic column, size 4, using isopropanol: water (4:1). The eluate was collected in 14 to 15 ml fractions by use of an automatic fraction collector set for a 15-minute collection period. Optical density measurements showed concentration of the 317 mμ absorbing material in tubes 65 to 88. The contents of these tubes were combined and the solvent evaporated yielding 254 mg of tan residue. On paper chromatograms this residue yielded a single well-defined light yellow spot at R_f 0.69 in butanol: acetic acid: water (4:1:5). In water saturated with butanol, the yellow spot appeared at R_f 0.59 but an additional spot which fluoresced blue in the presence of ammonia and UV light appeared at R_f 0.38.

The 254 mg of tan residue was further chromatographed on the LKB pressurized paper chromatographic column in water saturated with butanol. A sharp peak of the 317 mμ-absorbing component occurred in fractions 51 to 59. These were pooled and evaporated to dryness in vacuo, using a Rinco evaporator, yielding 78 mg of glassy tan solid residue. This material appeared chromatographically pure in several solvent systems. Analysis showed 52.42 %





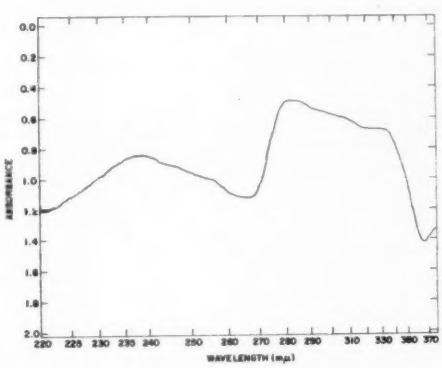


FIG. 1 (*top*). UV absorption spectrum of IAA oxidase inhibitor (25 ppm in ethanol).

FIG. 2 (*center*). IR absorption spectrum of IAA oxidase inhibitor (KBr pellet).

FIG. 3 (*bottom*). UV absorption spectrum of flavonol liberated by acid hydrolysis of inhibitor (15 ppm in ethanol).

TABLE I

EFFECT OF FLAVANOIDS ON IAA OXIDASE

FLAVANOID	(ppm)	DCP (m/l)	% IAA DESTROYED AFTER 60 min
...	...	...	26
...	...	5×10^{-5}	56
Natural Inhibitor	400	"	2
"	200	"	62
Kaempferol	15	"	0
"	6	"	51
Kaempferide	300	"	6
"	150	"	28
Rhamnocitrin	1500	"	0
"	300	"	40

carbon, 5.12 % hydrogen, and no nitrogen. The UV absorption spectrum (fig 1) showed λ-max at 317 mμ (E^{1} % 1 cm = 3.88 × 10^2) and at 268 mμ (E^{1} % 1 cm = 3.04 × 10^2). The infrared absorption spectrum (fig 2) showed a band characteristic of OH at 2.94 μ, saturated CH absorption at 3.4 and 3.48 μ, possibly α,β- unsaturated carbonyl absorption at 5.9 μ, aromatic-CH = CH- in the 6 μ region, and a broad band at 9.3 μ, possibly due to a -CO- group. This substance at 400 ppm completely inhibited IAA destruction in the standard enzyme assay (table I).

HYDROLYSIS OF INHIBITOR: The purified inhibitor, 16.1 mg, and 6 ml 1 % H_2SO_4 were heated on a steam bath for 1.5 hours. During the heating a dark yellow colored flocculent precipitate separated from the solution. The suspension was chilled in ice and the precipitate collected by centrifugation. After washing with water and drying in vacuo 2.3 mg of solid were obtained, mp 228 to 262 C. Recrystallization from a water-ethanol solution gave a light yellow colored product which turned brown and softened at 255 C, then melted with decomposition at 268 to 272 C.

TABLE II

COMPARISON OF R_f VALUES OF INHIBITOR HYDROLYSIS PRODUCT WITH THOSE OF KAEMPFEROL

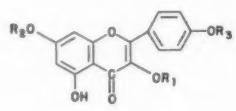
	FLAVONOL FROM PEA TIPS	KAEMPFEROL
Propanol : NH_4OH (7 : 3)	0.11	0.12
m-Cresol : acetic acid : H_2O (50 : 2 : 48)	0.58	0.55
Phenol : H_2O (80 : 20)	0.70	0.67
(88 : 12)	0.77	0.77
Butanol : acetic acid : H_2O (4 : 1 : 5)	0.90	0.91
15 % Acetic acid	0.07	0.06
2,6-Lutidine	0.97	0.97
Petroleum ether : benzene : ethyl acetate (45 : 45 : 20)	0.78	0.81

The UV absorption spectrum of this material (fig 3) was similar to that reported for kaempferol (7), λmax at 367 mμ (ε = 2.6 × 10^4) and 266 mμ (ε = 2.11 × 10^4). In table II are compared the R_f values of the isolated material in nine different solvent systems to those of authentic kaempferol (from Mann Research Laboratories, N. Y.).

In another run the acid hydrolysate was extracted with ethyl acetate before further workup. The ethyl acetate in turn was extracted with 15 % sodium bicarbonate solution. The bicarbonate extract was acidified with concentrated HCl, extracted with ethyl acetate, then the ethyl acetate removed in vacuo. By UV absorption spectra, paper chromatography, and cofactorial behavior in the IAAO assay it was demonstrated that the residue contained *p*-hydroxycinnamic acid. The 317 mμ λ-max of the inhibitor (fig 1) is probably due in part to the strong absorption at 310 mμ by the *p*-hydroxycinnamoyl group.

Paper chromatographic analysis of the aqueous portion of the acid hydrolysate, after extraction with ethyl acetate, indicated the presence of a single sugar. A phenylosazone of this sugar was prepared (8) which, after three recrystallizations from ethanol, melted at 203 to 204 C. As the melting point of this material was not depressed by an authentic sample of the phenylosazone of glucose, it is concluded that the only sugar released on acid hydrolysis of the IAAO inhibitor is glucose.

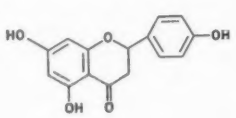
INHIBITORS: Inhibition of the IAAO system by the natural inhibitor, by kaempferol I (R_1=R_2=R_3=H), by kaempferide I (R_1=R_2=H; R_3=CH_3), and by rhamnocitrin I (R_1=R_3=H; R_2=CH_3) are compared in table I.



I

On a weight basis kaempferol is at least 30 times as effective as is the natural inhibitor. Kaempferol is also about 20 times as active as kaempferide and at least 100 times as active as rhamnocitrin.

FLAVANOID COFACTORS: The flavanone, naringenin (II), and flavone derivative, apigenin-7-glucoside (III), failed to inhibit IAAO, thus denying the possibility that inhibition of IAAO by flavanoids might be due to effects of meta hydroxyls at five and seven positions. Furthermore, II and III were moderately good cofactors for bean root IAAO.



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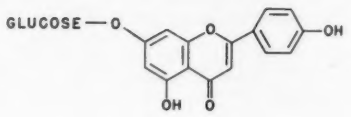


III

The relative effectivenss of DCP, naringenin, and apigenin-7-glucoside as cofactors for IAA destruction by bean root IAAO is shown in figure 4. The initial lag with apigenin-7-glucoside may mean that a small percentage of inhibitor was present, as the compound was not purified before use. Naringenin also failed to show any cofactor activity before two recrystallizations from aqueous ethanol.

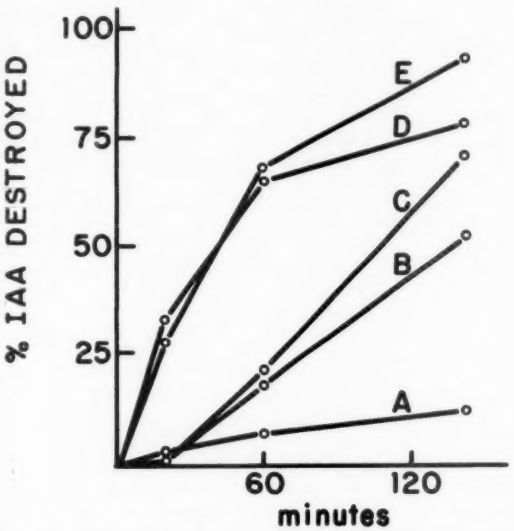
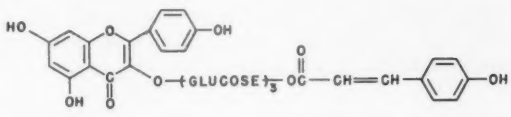


FIG. 4 (*lower right*). Effect of cofactors on IAA oxidase from bean roots. A = enzyme, 2×10^{-4} M IAA, 1×10^{-4} M manganous chloride. B = A + 1×10^{-4} M apigenin-7-glucoside. C = A + 2×10^{-5} M naringenin. D = A + 1×10^{-4} M naringenin. E = A + 5×10^{-5} M 2,4-dichlorophenol.

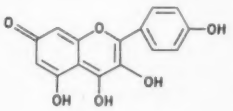
DISCUSSION

An IAA oxidase inhibitor has been isolated from the terminal buds of etiolated pea plants exposed to red light, which on hydrolysis yields kaempferol, glucose, and *p*-hydroxycinnamic acid. Structure studies, which will be reported in detail later, have shown that the kaempferol: glucose: *p*-hydroxycinnamic acid ratio in the inhibitor is 1:3:1 and have indicated that the compound is structure IV.

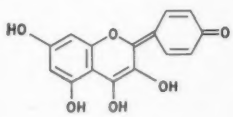


IV

Kaempferol, itself, is a considerably better IAA oxidase inhibitor than the kaempferol derivative isolated from the tips of pea plants. This is reasonable since the naturally occurring inhibitor appears conjugated with *p*-hydroxycinnamic acid, which is known to be an excellent IAA oxidase cofactor (3). Rhamnocitrin which has the 7-hydroxyl methylated, and kaempferide, which has the 4′-hydroxyl methylated, are much less active inhibitors than kaempferol. This suggests that kaempferol may be inhibiting the IAA oxidase system through its tautomeric structures, V and VI, which cannot exist, respectively, when the 7- and 4′-hydroxyls are methylated. Structures V and VI bear similarity to catechol and caffeic acid, excellent IAA oxidase inhibitors (3).



V



VI

Naringenin, II, and apigenin-7-glucoside, III, are cofactors for the IAAO system. Since it has been established that naringenin, apigenin, and kaempferol exist in the terminal buds of at least one plant species (5), it is tempting to speculate that one way in which the red-far red photoreaction affects plant growth is through control of IAA oxidase cofactor-inhibitor balance. Thus activation of the red-far red pigment by red light may lead to the triggering of the oxidative enzyme systems which convert IAAO cofactors such as naringenin, apigenin, or related compounds to the kaempferol derivatives that serve as IAAO inhibitors. It must be recognized, of course, that operation of IAAO in vivo, is not yet proved, though work of Goldacre (2) with pea stem sections suggests it.

SUMMARY

I. An inhibitor of IAA oxidase has been isolated from the buds of etiolated pea plants which were exposed to red light prior to harvest. This inhibitor is more abundant in red light treated buds than it is in completely dark grown controls. Structure studies indicate that the compound is a 3- (*p*-hydroxycin-

namoyltriglucosyl) derivative of kaempferol. In a standard assay this flavonol derivative inhibits IAA oxidase 100 % at 400 ppm.

II. A new class of compounds, 3,7-dihydroxyflavones, inhibitory for IAA oxidase of bean roots and etiolated pea buds has been discovered. Kaempferol, the most active member of this class yet tested, shows 100 % inhibition at 5×10^{-6} M in the standard assay.

III. Two flavonoid cofactors for IAA oxidase, naringenin and apigenin-7-glucoside, have been found.

IV. It is proposed that one possible mechanism for the effects of red light on plant growth is through control of IAA oxidase cofactor-inhibitor balance.

Acknowledgments

We thank Professor A. W. Galston, Yale University, for his advice and encouragement during this work. A helpful discussion was also held with Dr. Masaki Furuya, Yale University, who has been working with a similar inhibitor from the green leaves of Alaska peas.

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ASSIMILATION OF C^{14}, N^{15} LABELED UREA BY EXCISED APPLE & PEACH LEAVES [1, 2]

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The widespread use of urea as a nutrient spray is a reflection of its capacity to be absorbed and metabolized by the foilage of many plant species. Certain fruit crops, such as the peach, however, do not efficiently utilize nitrogen from foliar-applied urea (3, 7). It is not known whether the problem is one of absorption or subsequent metabolism of the applied urea. Experiments using urea-C^{14} (23) indicated assimilation of the carbon portion of urea but gave no conclusive evidence for assimilation of urea nitrogen. Evidence supporting assimilation of urea nitrogen in apple (2), citrus (11), coffee, cacao, and banana (4) is largely based on quantitative differences in leaf amino acid concentration following foliar application of urea. Such studies do not distinguish between the nitrogen derived from urea or that derived from proteolysis and consequently may not be unequivocal evidence for nitrogen absorption. This distinction can be made by incorporating the N^{15} isotope into the urea supplied. In view of the gross difference in utilization of foliar-applied urea, this study was conducted utilizing C^{14},N^{15} labeled urea to compare assimilation by apple and peach leaves.

MATERIALS & METHODS

To circumvent difficulties resulting from differential cuticular absorption, C^{14},N^{15} labeled urea was supplied through the petiole. Fifty young peach [*Prunus persica* (L.) Batsch, var. Elberta] and apple (*Pyrus Malus* L. var. Delicious) leaves were placed individually in 2 ml of 0.05 M phosphate buffer containing 3 mg urea (98.7 % N^{15} and 20μc urea C^{14} specific activity 1 μc/μmole) per ml. The leaves were illuminated for 20 hours with fluorescent and incandescent lights after which they were quick frozen and stored at −18 C until analyzed. Peach and apple leaves and watermelon (*Citrullus vulgaris* Schrad) seedlings also received urea C^{14} or urea N^{15} during a 12 hour absorption period in preliminary experiments.

The frozen leaves were extracted with 80 % (v/v) ethanol. A protein hydrolyzate of the ethanol insoluble residue was prepared according to Block et al (1). The ethanol extract was reduced in vacuo at 40 C and extracted with diethyl ether. The aqueous phase was purified for chromatography by ion exchange according to the method of Plaisted (15).

The purified extracts were subjected to two dimensional descending paper chromatography, employing phenol-water (4:1) and *n*-butanol-propionic acid-water (125:59:83) on 18 × 22 inch sheets of Whatman No. 1 filter paper. The amino acids were located by dipping the dry chromatograms in 0.25 % (w/v) ninhydrin in acetone containing 5 % (v/v) glacial acetic acid and heating for 30 minutes at 60 C in an ethanol atmosphere. Radioautograms were prepared from the paper chromatograms in the usual manner.

The amino acids of the purified extracts were also separated by ion exchange column chromatography by modifying the procedure of Moore et al. (13). One-half milliliter of each fraction was plated and counted for C^{14} on aluminum planchets. The remaining one-half ml of each fraction was analyzed for amino acids by the photometric ninhydrin procedure of Rosén (17). An aliquot from the planchet corresponding to a ninhydrin and/or radioactivity peak was used to verify the identity and purity of the amino acids by one dimensional paper chromatography employing the solvent systems described above.

Nitrogen in the fractionated amino acid samples and in samples obtained during preparation and purification of the leaf sample was analyzed for N^{15} in the mass spectrometer of the Stable Isotopes Laboratory of North Carolina State College.

RESULTS

A preliminary experiment was performed to determine the quantity of leaf material and urea-N^{15} required to measure incorporation into the free amino acids. After a 12 hour absorption period, with 12 mg of urea-N^{15} supplied to ten apple leaves through the petiole, N^{15} was incorporated into all of the amino acids analyzed (table I). The greatest enrichment was found in glutamic acid plus glutamine and alanine. Valine was also highly enriched, but contained an unidentified contaminant. The quantity of alpha amino nitrogen present in ten leaves was insufficient to accurately determine N^{15} incorporation into the minor acids.

Absorption of the C^{14},N^{15} urea treating solution into apple leaves was essentially complete within 3 hours. Characteristic symptoms of urea injury ap-

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[2] Published with the approval of the director of research as paper No. 1250 of the Journal Series.

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TABLE I

INCORPORATION OF N^{15} FROM UREA INTO APPLE LEAF AMINO ACIDS AFTER 12 HOURS

AMINO ACID	ATOM % EXCESS N^{15}
Alanine	7.6
Asparagine plus serine*	4.6
Aspartic acid	4.8
Cysteine	0.4
Glutamic acid plus glutamine*	11.6
Glycine	2.8
Histidine	0.8
Hydroxyproline	1.3
Isoleucine	0.9
Leucine	1.2
Lysine	0.9
Proline	1.2
Threonine	3.3
Tyrosine plus phenylalanine*	0.1
Valine**	9.2

* Emerged from the column together.
** Contained an unidentified nitrogen containing compound.

peared after 9 hours and developed into necrotic areas by the end of 20 hours. Approximately two-thirds of the treating solution was absorbed by peach leaves during the first 10 hours. No symptoms of urea injury were observed on peach leaves during the absorption period.

The ion exchange purification of the peach and apple leaf extracts resulted in considerable retention of N, N^{15}, and C^{14} against the 4 N NH_4OH used to elute the amino acid fraction. It is unlikely that the substances retained were amino acids, since arginine, the most basic amino acid commonly present in plant materials, is quantitatively eluted by 4 N NH_4OH. Apple leaves incorporated a greater proportion of N^{15} and C^{14} from urea into the neutral and anion fraction (sugars & organic acids) than into the cation fraction (amino acids). The reverse was true for peach leaves.

Radioautography of the free amino acid fraction of apple leaves receiving C^{14},N^{15} labeled urea showed the presence of radioactivity in aspartic, glutamic, serine, and glutamine. The apple leaf extract contained a considerable quantity of non-hydrolyzed urea, as evidenced by a very heavily exposed area corresponding to urea by the *p*-dimethylaminobenzaldehyde test for the ureido group (1). The radioautograph of the apple leaf protein hydrolyzate indicated radioactivity in alanine, serine, asparatic, and glutamic acid.

The radioautograph of the free amino acids of peach leaves showed radioactivity in aspartic, glutamic, asparagine, glutamine, and proline with less in serine, glycine, threonine, alanine, tyrosine, and gamma aminobutyric acid. Arginine, leucines, phenylalanine, and pipecolic acid were present but did not contain sufficient radioactivity to expose the film after 4 weeks. Non-hydrolyzed urea was present on the radioautograph to a far lesser extent than in apple, indicating more extensive hydrolysis of absorbed urea. The peach leaf protein hydrolyzate radioautograph indicated aspartic acid labeled in addition to two unknown compounds not detected by ninhydrin or *p*-dimethylaminobenzaldehyde which chromatographed beyond phenylalanine in both solvents.

The radioautograph of the organic acid and sugar fraction of apple leaves receiving C^{14},N^{15} labeled urea indicated more radioactive compounds than were found in the case of peach. Compounds in this fraction such as organic acids serve as the carbon skeleton for amino acid synthesis. In view of the marked degree of urea hydrolysis which occurred in peach as compared to apple at the end of 20 hours, it is feasible that more complete turnover of organic acids, or a different type of assimilation, had occurred in peach resulting in less C^{14} activity remaining in the organic

TABLE II

INCORPORATION OF N^{15} & C^{14} FROM UREA INTO APPLE LEAF AMINO ACIDS

AMINO ACID	N^{15} ATOM % EXCESS	C^{14}/N^{15} cpm/μg N^{15}	C^{14}/N^{15} (AMINO ACID) / C^{14}/N^{15} (UREA)*
Arginine	3.2	30	0.080
Asparagine plus serine**	8.8	730	1.95
Aspartic acid	9.8	23	0.062
Cysteine	1.1	24	0.064
Glutamic acid plus glutamine**	9.4	1,090	2.90
Leucines	2.2	7	0.018
Lysine	1.5	27	0.072
Proline	6.7	519	1.38
Threonine	10.6	807	2.15
Tyrosine plus phenylalanine**	5.4	104	0.277
Protein hydrolyzate	5.7	48	0.128
Ether soluble fraction	5.5	318	0.850

* The C^{14}/N^{15} ratio of the urea supplied was 375 cpm per μg N^{15}.
** Emerged from the column simultaneously.

acids. This is supported, in part, since the amino acids of peach were more highly labeled than the organic acids. The high reserve of labeled urea undergoing gradual hydrolysis in apple leaves would supply C^{14} for organic acid synthesis and would be expected to have a greater C^{14} content in the organic acid pool. The organic acids of apple were much more highly labeled than the amino acids. The results of a 12 hour absorption study with apple and peach leaves were similar. Watermelon seedlings receiving urea-C^{14} exhibited high activity in aspartic acid, glutamic acid, glycine, gamma aminobutyric acid, alanine, citrulline, glutamine, arginine, and urea. The urea spot was only moderately radioactive, indicating considerable hydrolysis had occurred. Our results for watermelon agree with those of Kasting and Delwiche (10) except that proline was not found to be highly radioactive.

The data for urea assimilation in apple leaves after 20 hours' absorption is given in table II. The high N^{15} enrichment found in aspartic and glutamic acids and their amides is typical of nitrogen assimilation via ammonia. The incorporation of N^{15} among the other amino acids indicates considerable distribution of assimilated urea nitrogen. It is difficult to account for the high N^{15} enrichment found in threonine. The C^{14}/N^{15} ratios for the amino acids (column 2) reflect the extent of dilution these isotopes experienced during urea assimilation. The ratio of isotopes in the amino acids to that of the urea supplied (column 3) more clearly indicates the degree of equilibration which occurred during assimilation. Ratios in the third column approaching unity would suggest assimilation of $N^{15}H_3$ and $C^{14}O_2$ from ammonia and carbon dioxide pools of similar equilibrium. The low ratio found for arginine argues against an alternative explanation for a unity ratio, namely, urea assimilation prior to hydrolysis as postulated for Chlorella (8, 20). Unfortunately the isotope data represent the entire molecule and not individual C–N or N–C–N groups within the amino acids. Thus, ratios approaching two found for asparagine plus serine and for threonine do not necessarily represent assimilation of urea prior to complete hydrolysis, as suggested by Webster (22). Assimilation of urea carbon into glutamic acid plus glutamine was nearly three times that for urea nitrogen.

The relatively high C^{14}/N^{15} ratios of several of the apple leaf amino acids, the protein hydrolyzate and the ether soluble fraction suggest net synthesis of nitrogenous compounds during the 20 hour absorption period.

Urea assimilation into peach leaf amino acids was found similar to apple, but more extensive equilibration of isotopes was evident (table III). Amino acids with high N^{15} enrichment were also highly labeled with C^{14}.

Several unidentified compounds were obtained during the column chromatography of the amino acid fraction of apple and peach leaves which were highly labeled with N^{15} and C^{14}.

Discussion

Apple and peach leaves supplied C^{14}, N^{15} labeled urea through the petiole readily assimilated both isotopes into amino acids, amides, proteins, and ether soluble compounds within 20 hours. Only a small fraction of the urea absorbed by peach leaves remained in the leaf as non-hydrolyzed urea. Most of the

TABLE III

INCORPORATION OF N^{15} & C^{14} FROM UREA INTO PEACH LEAF AMINO ACIDS

AMINO ACID	N^{15} ATOM % EXCESS	C^{14}/N^{15} cpm/μg N^{15}	C^{14}/N^{15} (AMINO ACID) C^{14}/N^{15} (UREA)*
Alanine	11.7	13	0.035
Arginine	4.0	9	0.024
Asparagine plus serine**	13.7	24	0.064
Aspartic acid	14.3	12	0.032
Cysteine	0.9	21	0.056
Gamma aminobutyric acid	3.2	118	0.315
Glutamic acid	12.5	24	0.064
Glutamine plus proline**	15.2	26	0.069
Histidine	4.4	65	0.174
Leucines	2.6	115	0.306
Lysine	2.2	32	0.085
Pipecolic acid	1.8	42	0.112
Threonine	6.1	56	0.150
Tyrosine plus phenylalanine**	5.1	127	0.312
Valine	1.8	35	0.093
Protein hydrolyzate	5.3	24	0.064
Ether soluble fraction	4.1	39	0.104

* The C^{14}/N^{15} ratio of the urea supplied was 375 cpm per μg N^{15}.
** Emerged from the column simultaneously.

C^{14} and N^{15} present in the water soluble fraction of apple leaves was in the form of urea. Our results of C^{14} and N^{15} incorporation from labeled urea into apple and peach leaf amino acids indicate assimilation after hydrolysis to $C^{14}O_2$ and $N^{15}H_3$ and support the conclusion of Webster et al. (23) for urea assimilation in beans. These workers arrived at their conclusion on the basis of similarity of C^{14} assimilation from urea-C^{14} and $NH_4HC^{14}O_3$. The preponderance of C^{14} and N^{15} in the amino acids, in particular glutamate, aspartate and their amides, and alanine, glycine, serine, and threonine suggest $C^{14}O_2$ fixation and active transamination of $N^{15}H_3$ with the respective alpha-keto analogs of the amino acids. Fixation of $C^{14}O_2$ was evident in the radioautograms of the organic acid fraction of apple and peach leaves receiving C^{14}, N^{15} labeled urea.

The initial urea hydrolysis step appears to be limiting urea assimilation in apple leaves in view of the high reserve of non-hydrolyzed urea and the high C^{14}/N^{15} ratio in glutamic acid plus glutamine and asparagine. As urea is hydrolyzed or, in some manner initially assimilated, both the carbon and nitrogen portions accumulate in these amino acids and eventually are distributed to other amino acids. With peach leaves, the initial hydrolysis step does not appear to be limiting assimiliation. The distribution pattern of C^{14} and N^{15} in peach leaf amino acids indicates efficient urea assimilation. Urease, the enzyme catalyzing urea hydrolysis, has been reported in these plants (5), supporting the evidence for assimilation via ammonia and carbon dioxide found in the present investigation.

Urea assimilation in apple and peach leaves agrees in a general way to that found by Kasting and Delwiche (10) for watermelon, Boynton et al. (2) for apple, and Cain (4) for coffee, cacao, and banana in that aspartic and glutamic acid and their amides are important intermediates. Urea nitrogen was assimilated in apple and peach leaves in a manner typical of ammonia assimilation in tobacco (19) and tomato (12).

The efficient utilization of urea by peach leaves found in this study is in contradiction to results which indicated absorption but subsequent inability to utilize foliar-applied urea (7). The discrepancy observed may be a result of the manner in which urea was supplied to the leaves rather than a difference in urea assimilation. In the present investigation urea was supplied through the petiole rather than applied to the foliage surface, and therefore was absorbed into the transpirational stream and distributed throughout the leaf. This technique obviates the initial requirement of cuticular penetration and provides the leaf ready access to assimilate urea. Historically, peach trees have responded only to excessively high applications of urea (3, 6, 14, 16). It is difficult to evaluate the results of certain of the cited reports, since a portion of the foliar-applied urea may have dripped or been washed from the leaves and subsequently absorbed through the roots, resulting in a nitrogen response quite apart from that which would have occurred in the absence of root absorption. Soil applications of urea to peach trees have been found beneficial (24) and it is possible that a portion of urea so applied enters the plant as urea in view of evidence presented by Wallace and Ashcroft (21) that urea is absorbed by lemon and bean roots prior to hydrolysis. Foliar applications of urea to pear trees have met with little success (9, 16), yet it has been shown that pear trees utilize urea injected into the branches (18). The similarity in response to foliar-applied urea by peach and pear suggests the problem in both may be one of absorption, not utilization.

Summary

Results of this investigation demonstrate utilization of urea by excised apple and peach leaves. When C^{14}, N^{15} labeled urea was supplied through the petiole, both isotopes were readily incorporated into amino acids, amides, and protein within 20 hours. Urea hydrolysis proceeded to a greater extent in peach than in apple leaves, as evidenced by residual urea and comparison of isotope incorporation into leaf constituents.

The results of C^{14} and N^{15} incorporation from labeled urea into apple and peach leaf amino acids indicate assimilation after hydrolysis to $C^{14}O_2$ and $N^{15}H_3$. The preponderance of C^{14} and N^{15} in the amino acids, in particular glutamate, aspartate and their amides, and alanine, glycine, serine, and threonine is a reflection of $C^{14}O_2$ fixation and active transaminations of fixed $N^{15}H_3$ with the respective alpha-keto analogs of the amino acids.

The failure of peach leaves to effectively utilize foliar-applied urea is not due to their inability to hydrolyze nor subsequently assimilate urea, but perhaps is due to difficulty in foliar absorption.

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EFFECT OF SOIL MOISTURE STRESS ON ABSORPTION & TRANSLOCATION OF PHOSPHORUS APPLIED TO LEAVES OF SUNFLOWER [1, 2]

A. M. WILSON & CYRUS M. McKELL [3]

Plants subjected to moisture stress undergo physiological adjustments which result in a reduction or cessation of growth. The effect which moisture stress may have on the movement of substances from leaves to regions of growth or storage, needs further clarification. In addition, the influence of moisture stress on cuticle permeability must be considered in problems dealing with the effectiveness of substances applied to leaves. Weintraub et al. (10) showed that leaves of wilting bean plants absorb less 2,4-dichlorophenoxyacetic acid (2,4-D) than do plants with adequate soil moisture. The effect of moisture stress on the subsequent translocation of 2,4-D was not measured. Pallas (5) found no effect of moisture stress on absorption of 2,4-D by bean leaves. The effect of moisture stress on translocation of the herbicide was estimated by assaying radioactivity in two 1-cm sections of the epicotyl. As the soil approached the permanent wilting percentage translocation of 2,4-D decreased to approximately half that found near field capacity. It is not clear, however, whether moisture stress caused a decreased transport of 2,4-D or altered the distribution of the translocated herbicide.

Koontz and Biddulph (3) measured the effects of various factors on absorption and translocation of phosphorus applied to bean leaves. Results obtained by Dybing and Currier (2) indicate that phosphate penetrated the cuticle of Zebrina more rapidly than other chemicals tested. However, stomatal penetration of surfactant-free aqueous solutions was not clearly demonstrated. Biddulph (1) showed that movement of phosphorus from a treated bean leaf occurred in the phloem. From analyses of squash phloem exudate by Tolbert and Wiebe (9) it has been demonstrated that inorganic phosphate was the principal translocated form.

We undertook the present work to measure the effect of soil moisture stress on the absorption of phosphorus by a mature leaf, the transport of phosphorus from the treated leaf, and the distribution of phosphorus to other plant parts. Of particular interest was the effect of moisture stress on the rate of accumulation of phosphorus in the more rapidly growing regions.

Materials & Methods

We used a dwarf form of the sunflower plant (*Helianthus annuus* L.). Plants were grown in 550 g of Yolo fine sandy loam in a controlled environment: Daylength of 16 hours, light intensity of 1,000 ft-c, temperature of 21 C, and relative humidity of 75 to 90 %. Soil moisture stress was maintained below 0.3 atmosphere until the drying cycle was initiated. Water was withheld in sequence so that desired tensions would be reached approximately three weeks after the seedlings emerged. During the single drying cycle, soil moisture percentage was estimated daily from container weights. When the desired tensions were reached, 25 μl of P^{32}-labelled NaH_2PO_4 (10 mM, pH 5, surfactant-free aqueous solution) were placed on a mature leaf in droplet form. Percentage moisture at harvest was obtained from a moisture determination on soil samples. This value was corrected for transpiration loss during the 24-hour treatment period to give a more accurate measure of percentage moisture at treatment. A soil moisture characteristic curve was used to convert percentage moisture to soil moisture tension in atmospheres (6). After the absorption period, the treated leaf was detached and washed with 0.01 N HCl containing 0.01 % sodium heptadecyl sulfate. The remainder of the plant was separated into root, stem, mature leaves, and growing tip. For convenience, the shoot apex and young expanding leaves will be referred to as the growing tip. Plant parts were digested in HNO_3 and aliquots taken for radioassay. The sum of radioactivity in plant parts and wash solution was used to calculate the amount of phosphorus absorbed and translocated.

Adequacy of the washing technique was checked by collecting the wash solution from treated second leaves in four successive 25 ml portions. The amount of phosphorus in the four successive portions, expressed as a percentage of the total washed from the leaf, was 99.25, 0.66, 0.06, and 0.03. Adding phosphate to the wash solution did not alter measurably the apparent absorption nor did it change the distribution of radioactivity in the four portions washed from the leaf. Results suggest that there is an insignificant amount of phosphorus leached from the leaf during washing and that errors due to the absorption of phosphorus to the external surface of the cuticle are small.

[1] Received June 19, 1961.

[2] Cooperative investigations of the Crops Research Division, ARS, USDA, and the University of California, Davis.

[3] Plant Physiologists, Crops Research Division, ARS, USDA. Present address of second author: Department of Agronomy, University of California, Riverside.

Results

Absorption and translocation of phosphorus were measured at three levels of soil moisture stress after a 24-hour absorption period. Moisture stress decreased phosphorus absorption (table I). Plants at 0.3 atmosphere absorbed 80 % of the applied phosphorus as compared with an absorption of 33 % by plants at 5 atmospheres and 15 % at 16 atmospheres.

Table I

Effect of Soil Moisture Stress on Phosphorus Absorption & Translocation*

Tension (atm)**	Phosphorus absorbed (%)***	Absorbed phosphorus translocated (%)***	Total phosphorus translocated (μg)***
0.3±0.0	80	16	0.95
5±2	33	6.9	0.18
16±1	15	6.4	0.08

* Phosphorus was applied to one of first leaves. Absorption period was 24 hours. Plants were 20 to 21 days old at treatment.

** Soil moisture tensions at time of treatment. Values are means of three replications and their standard deviations. The corresponding tensions at harvest were 0.5 ± 0.1, 8 ± 3, and 20 ± 1 atmospheres.

*** LSD at 0.05 level is 23 for percentage absorbed, 1.3 for percentage translocated, and 0.30 for μg translocated.

Not only was more phosphorus absorbed under favorable moisture conditions, but a higher percentage of the absorbed phosphorus was translocated from the treated leaf. Plants at 0.3 atmosphere translocated more than 16 % of the phosphorus which had been absorbed as compared to less than 7 % for plants at 5 and 16 atmospheres. Soil moisture stress also altered the distribution of the translocated phosphorus among the plant parts (table II). The amount of phosphorus in the growing tip, stem, and root was expressed as a percentage of the amount translocated. In plants at 0.3 atmosphere 55 % of the phosphorus was found in the growing tip as compared with 20 % in the stem. At 5 atmospheres the amount of phosphorus in the growing tip was about equal to the amount in the stem. At 16 atmospheres the amount of phosphorus in the stem exceeded that in the growing tip. The relative amount of phosphorus in the root was about the same for each moisture stress.

Table II

Effect of Moisture Stress on Distribution of Translocated Phosphorus*

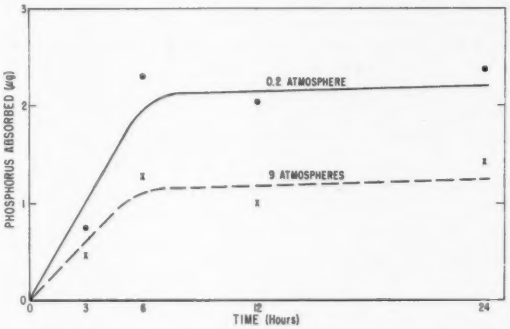
Tension (atm)	% Translocated phosphorus in plant parts**		
	Growing tip***	Stem	Root
0.3±0.0	55	20	24
5±2	37	39	22
16±1	28	43	26

* Conditions are the same as described below table I.

** LSD at 0.05 level = 12 %.

*** Growing tip includes portion of plant above second node.

The rate of phosphorus absorption, translocation, and accumulation was measured at two moisture stress levels using four absorption times. The decreased absorption of phosphorus at high moisture stress was due to a low initial rate of entry (fig 1). In the first 6 hours 2.1 μg of phosphorus were absorbed by plants at 0.2 atmosphere as compared with 1.1 μg absorbed by plants at 9 atmospheres. Although moisture from the applied solution was no longer visible after about 45 minutes, rapid absorption of phosphorus continued for 6 hours. The sharp decline in absorption rate after 6 hours was probably due to the nearly complete



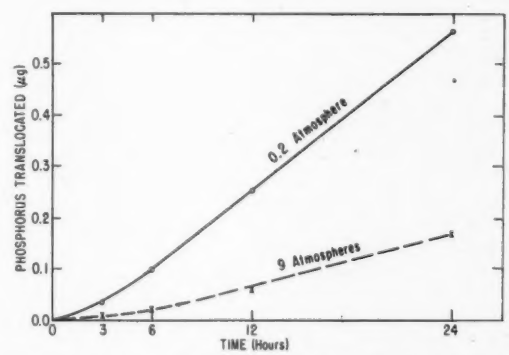


Fig. 1. Effect of moisture stress on amount of phosphorus absorbed by a treated second leaf. Plants were harvested after an absorption period of 3, 6, 12, and 24 hours. Average soil moisture tensions and their standard deviations were 0.2 ± 0.1 and 9 ± 3 atmospheres at treatment. Relative humidity was controlled to 60 ± 2 % during the absorption period. Plants were 24 to 25 days old at treatment.

Fig. 2. Effect of moisture stress on the amount of phosphorus translocated from a treated second leaf. Conditions are the same as described under figure 1.

evaporation of the water film surrounding the phosphate ions (3). Availability of soil moisture to plants at 0.2 atmosphere apparently did not slow down the rate of dehydration of the applied phosphorus. After the first 6 hours the amount of phosphorus translocated from the treated leaf was approximately linear with time (fig 2). The calculated rates of phosphorus transport were 0.026 μg per hour at 0.2 atmosphere and 0.008 μg per hour at 9 atmospheres. The time required for phosphorus to penetrate the cuticle, enter the phloem, and move down the petiole would account for the low translocation rates during the first few hours. The accumulation of translocated phosphorus in the various plant parts was expressed as μg phosphorus per gram dry weight. After 24 hours the concentration of phosphorus in plants at 0.2 atmosphere was more than two times higher in the growing tip than in the stem (fig 3). In plants at 9 atmospheres the phosphorus accumulation in the growing tip was not significantly higher than in the stem. The movement of phosphorus into leaves appeared to be related to leaf age and availability of soil moisture. The third leaves accumulated more phosphorus than did the more mature second and first leaves. Radioautograms showed that root tips of plants at 0.2 atmosphere accumulated more phosphorus than did root tips of plants at 9 atmospheres.

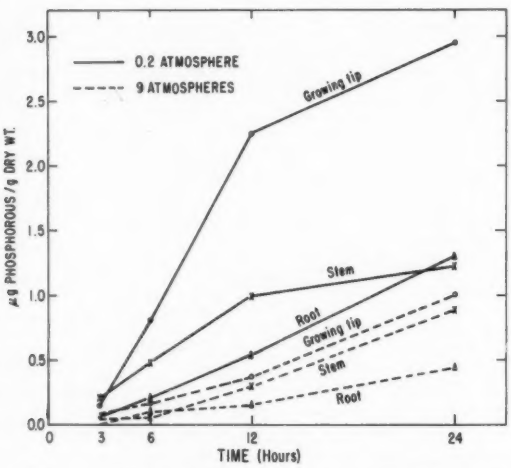


FIG. 3. Effect of moisture stress on accumulation of phosphorus in growing tip, stem, and root. Conditions are the same as described under figure 1. The growing tip includes the portion of plant above fourth node.

DISCUSSION

In this study soil moisture stress increased during the treatment period as indicated in the legend to table I. However, the internal stress to which the plants were subjected undoubtedly did not follow the same pattern. During low transpiration rates at night, water uptake may have equaled or exceeded water loss and resulted in a leveling off or decrease in moisture stress in the plant (8). Measurement of phosphorus absorption and translocation rates in plants treated at various times during the day and night would be needed to reveal these diurnal fluctuations in moisture stress.

Stomatal closure at high moisture stress may explain in part the decreased phosphorus absorption. But since stomatal penetration of surfactant-free aqueous solutions has not been clearly demonstrated (2), the importance of stomatal opening in this study was probably small.

The development of a thicker cuticle by plants exposed to moisture stress is another possible factor in the reduced absorption of phosphorus. Skoss (7) found heavier cuticles on leaves of *Nicotiana glauca* plants subjected to a series of drying cycles. It is uncertain, however, whether or not a thicker cuticle would have developed in 3 or 4 days during the single drying cycle used in the present study.

The degree of cuticle hydration may have influenced its permeability to a greater extent than increased deposition of cutin and wax during the drying cycle. The permeability of plant cuticle apparently adjusts to changes in the internal water balance and to fluctuations in the humidity of the environment. A water deficit in the leaf, with the consequent dehydration of cutin, may restrict cuticular transpiration as well as the inward diffusion of substances applied to leaves (4).

Moisture stress undoubtedly influences the movement of phosphorus from treated leaves in a number of ways. Temperature effects, inhibitor sensitivity, and energy requirements indicate that active accumulation is involved in the movement of phosphorus into cells (11). Because phosphorus moves against a concentration gradient into sieve elements, an active secretion mechanism for this process has also been suggested (12). Moisture stress on cell metabolism may slow down the rate of phosphorus accumulation by leaf parenchyma cells and restrict its secretion into the phloem.

Turgor pressure gradients in the phloem would be affected more directly by moisture stress. Obviously, a degree of moisture stress which limits water absorption by roots and, consequently, xylem transport of water to the leaf will also limit the rate of phloem transport from the leaf.

More difficult than explaining the decreased translocation rate in plants undergoing moisture stress is the attempt to understand why phosphorus moved at all from wilting plant leaves. Secretion of sucrose into the phloem against a concentration gradient would permit the sieve elements to remain turgid even when the surrounding cells are flaccid (13). The withdrawal of sugars, phosphorus, and other solutes from sieve elements of an expanding leaf may result in a lower turgor pressure in its sieve elements than would be found in older, more flaccid leaves. Phosphorus could thus move in the translocation

stream along turgor pressure gradients from a wilting leaf to a younger more turgid leaf.

At low moisture stress the high concentration of phosphorus in the growing tip as compared with the concentration in the stem suggests an ability of dividing and enlarging cells to accumulate phosphorus actively. A large part of the phosphorus in the stem is probably present in sieve elements and therefore available for accumulation by the growing tip. That this ability to accumulate phosphorus is partially lost as moisture becomes less available, is indicated (fig 3) by the small difference in the concentration of phosphorus in the stem and growing tip of plants at high moisture stress.

The reduced growth of plants exposed to moisture stress is undoubtedly brought about by the interaction of a large number of factors. Increase in dry weight, an important aspect of growth, frequently depends on phloem transport to an organ. The effects of moisture stress on the process of secretion into sieve elements, mass flow along hydrostatic pressure gradients, and active accumulation by cells will limit the rate at which substances are translocated to an organ and consequently reduce its rate of increase in dry weight. However, certain effects of moisture stress on translocation may be considered as secondary. The ability of a cell to accumulate substances from the phloem actively, for example, may depend on its metabolic activity and turgor pressure. Thus moisture stress, through its influence on metabolic activity and turgor pressure, would affect indirectly the rate at which substances are translocated to the cell.

The particular degree of moisture stress which would result in a reduced absorption and translocation of phosphorus may vary according to environmental conditions. A high transpiration rate during low humidity and high temperature may lead to an internal water deficit. Under these conditions decreased absorption and translocation may occur at comparatively low levels of soil moisture stress.

Summary

Leaves of a dwarf form of the sunflower plant were treated with P^{32}-labelled NaH_2PO_4 at various levels of soil moisture stress. After 24 hours plants at 0.3 atmosphere had absorbed 80 % of the applied phosphorus as compared with an absorption of 33 % at 5 atmospheres and 15 % at 16 atmospheres.

Moisture stress also resulted in less effective translocation of the absorbed phosphorus. In plants at 0.3 atmosphere 16 % of the absorbed phosphorus was translocated from the leaf in 24 hours as compared with less than 7 % in plants at 5 and 16 atmospheres. After an initial lag, the amount of phosphorus translocated from the treated leaf was approximately linear with time.

Moisture stress altered the relative distribution of the translocated phosphorus within the plant. Plants with adequate moisture accumulated a higher concentration of phosphorus in growing regions than did plants exposed to moisture stress. The effectiveness of phosphorus applications to leaves was reduced as availability of water to the plant was decreased by soil moisture stress.

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UPTAKE OF CALCIUM AND CHLORINE IN ROOTS OF ZEA MAYS[1, 2]

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In a previous paper (2) it was shown that uptake of Na by non-vacuolated tissue of the root tips of maize is independent of metabolism. The ability to absorb Na actively and to retain previously absorbed labeled Na against isotopic exchange was found to develop concomitantly with the appearance of well defined vacuoles. In the present work a similar relationship has been found with respect to the uptake of both Ca and Cl.

EXPERIMENTS

Dark-grown (26 C) tips of maize roots (*Zea mays* L., var. Peoria) were used. The plants were grown in 0.00025 N $Ca(NO_3)_2$ and the primary roots were cut into segments 0 to 1.8 mm, 1.8 to 3.8 mm, 3.8 to 7.8 mm, and 7.8 to 11.8 mm measured from the tip as described previously (2). The technique of periodic measurement of uptake over 24 hours by a single batch of 40 root segments was again used. The medium consisted as before of 500 ml of a 0.005 N solution of the appropriately labeled salt under investigation. The solutions were aerated continuously during the experimental period. The initial pH of 6.0 did not change during the experimental period. The large solution to tissue ratio was deemed advisable because of evidence that substances inhibitory to ion uptake are released by these roots. Investigation of this is proceeding and will be published separately.

The analytical procedure for Na has already been described (2). Because Cl^{36} and Ca^{45} are beta emitters the procedure for analysis of Ca and Cl had to be modified to eliminate errors due to self-absorption. When Cl uptake was to be determined the segments at the end of the 24 hour absorption period were extracted with four successive 2 ml portions of boiling water. The 2 ml extracts were evaporated in separate nylon planchets, counted using a TGC-2 Geiger tube (Tracerlab) and a Berkeley Decimal Scaler (Model 2001), and the aggregate count determined. Counting of extracted segments indicated that all of the Cl absorbed was removed by this procedure. The aggregate count was compared with the activity of 1.0 ml of the experimental solution to obtain an estimate of the Cl taken up by the tissue at the end of the experiment. The uptake of Cl for the other time intervals could then be calculated from the ratios of the activities of the fresh segments determined periodically during the experiment to that of the fresh material at the end of the experiment.

Estimation of Ca absorbed was accomplished similarly except that, at the end of the experiment, the segments were ashed (600 C for ½ hr) and taken up in hot dilute HCl before being evaporated in planchets. Because of the minute masses involved corrections for differential self-absorption were unnecessary.

The uptake of Na and Cl from 0.005 N NaCl at 26 C is shown in figure 1, uptake of Ca and Cl from 0.005 N NaCl at 26 C in figure 2, and uptake of Ca at 2 C and 26 C from 0.005 N $CaCl_2$ in figure 3.

The salts were analytical reagent grade. The water was laboratory distilled further purified by redistillation in all glass system. Ca^{45} and Cl^{36} were obtained from the Oak Ridge National Laboratory. Na^{22} was obtained from the Nuclear Science and Engineering Corp. Chemical determinations of Ca were made using cyclohexane diamino tetraacetic acid and calcein indicator (1).

DISCUSSION

Data in the graph (fig 1) indicate that in the meristematic region 0 to 1.8 mm from the root tip the uptake of Cl, like that of Na, is purely non-metabolic. In this segment equilibrium is attained much more quickly with respect to Cl distribution than with respect to Na distribution between the medium and the tissue. At equilibrium the Cl taken up is about 1/10 the value for Na. Rapid equilibration and relatively low uptake are characteristic of non-metabolic anion uptake and have been ascribed to the virtual absence of positively charged exchange sites within the tissue (3). In the segments farther from the tip the uptake of Cl parallels that of Na and evidently is largely metabolic. The non-metabolic uptake of Na in these older sections represents a considerable fraction of the total uptake, (2, fig 2). When this is taken into account it appears that Na and Cl are metabolically absorbed by these segments at about the same rate. Similar data for the absorp-

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[2] This paper is based on work performed under contract No. AT-(11-1)-34, Project 5, with the Atomic Energy Commission.

tion of Ca and Cl are presented in figure 2. Again, uptake of both cation and anion appears to be non-metabolic in the first segment although when the cation is calcium the capacity of the tissue for Cl uptake is considerably greater than in the case of Na. The usual effect of a slowly absorbed cation upon anion uptake is here reversed. The reason for this is not entirely clear but may be that the electronegative potential of the tissue is lowered significantly by the absorption of a divalent cation; the resistance of Cl entry is thereby reduced. This mechanism has been proposed by M. M. Elgabaly (article submitted to Plant & Soil). Other explanations are possible. For instance the water free space may be enlarged as a result of flocculation of protoplasm caused by Ca (6).

Equilibrium between the tissue and the surrounding medium with respect to the distribution of both Ca and Cl appears to be attained rapidly. Rapid equilibration with respect to Ca distribution has been reported by Moore et al. for excised barley roots (4). They suggest that non-metabolic uptake of Ca may occur mostly externally to the cytoplasm. Stimulating effects of Ca upon the respiration of the tip sections indicate, however, that more than the cell wall is involved in non-metabolic uptake of this tissue. Respiratory studies will be reported in a separate paper. The contrasting behavior of Na is, at any rate, quite striking; it illustrates the dependence of free space measurements upon the ion used. We think that living plant tissue cannot be considered merely as a reticulum lined with fixed negative charges; the extent of non-metabolic uptake for any cation may well depend upon the stability of chemical complexes formed within the protoplasm or upon the possibility of formation of essentially crystalline structures (5). Note that after 10 hours in $CaCl_2$ an apparent slight increase in the contents of both Ca

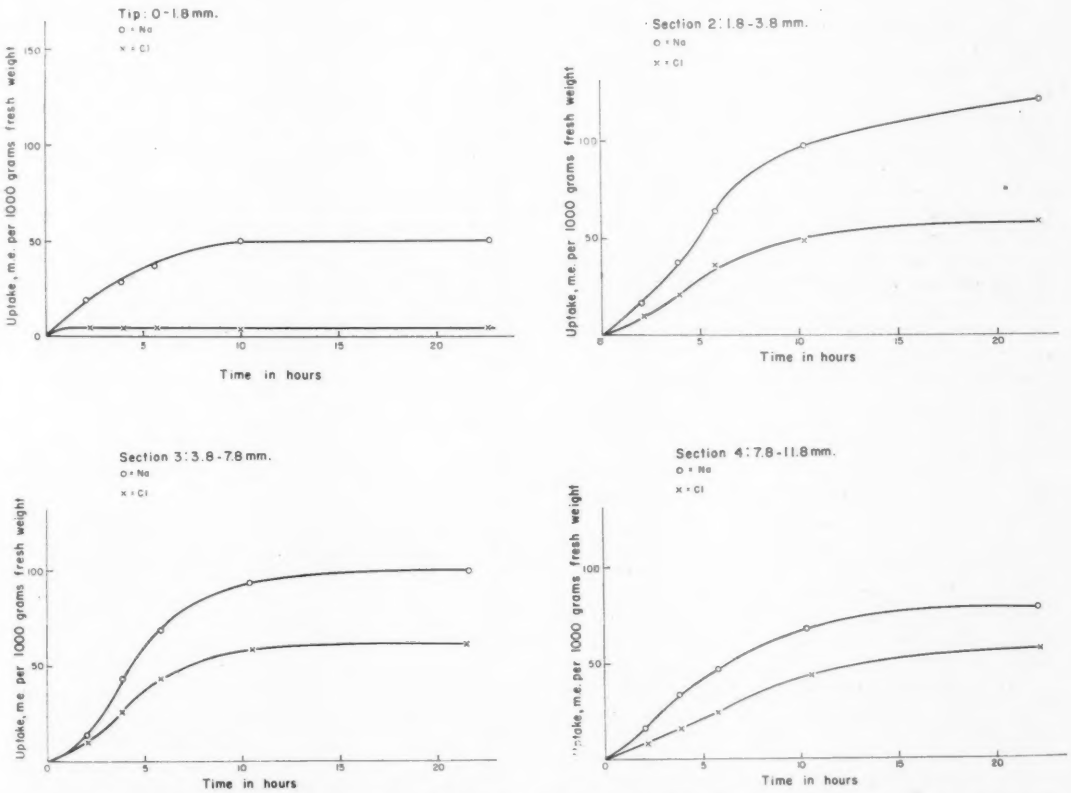
Tip: 0-1.8 mm.
O = Na
× = Cl
Uptake, m.e. per 1000 grams fresh weight
Time in hours
Section 2: 1.8-3.8 mm.
O = Na
× = Cl
Uptake, m.e. per 1000 grams fresh weight
Time in hours
Section 3: 3.8-7.8 mm.
O = Na
× = Cl
Uptake, m.e. per 1000 grams fresh weight
Time in hours
Section 4: 7.8-11.8 mm.
O = Na
× = Cl
Uptake, m.e. per 1000 grams fresh weight
Time in hours

FIG. 1. Sodium and chloride uptake versus time for various sections of maize roots at 26 C. For each curve, 40 segments of tissue were placed in 500 ml of 0.005 N NaCl labeled with 0.02 μc of Na^{22} or Cl^{36} per ml. The pH of the experimental solutions was 6.0. At the intervals indicated in each curve the segments were removed briefly from the medium, blotted dry, and counted. The uptake of sodium and chloride as meq/1000 g fresh weight was then calculated from the known fresh weight of the segments.

and Cl takes place. This has previously been noted by Moore et al. (4) in Ca uptake studies with excised barley roots. The significance of this is in doubt. Moore et al. suggest that during the experimental period development of the cells may occur resulting in increased capacity for non-metabolic uptake. Such development is apparently prevented in a NaCl medium. In view of the fact, however, that the same effect is seen at low temperatures (fig 3) it seems more likely that it is related to a physical effect of Ca ions upon the protoplasm.

In the older sections the uptake of Ca is apparently a metabolic process. This is apparent from the forms of the uptake curves and the quantities of Ca taken into the tissue as well as from the data of figure 3 where the uptake at 2 C is compared with that at 26 C. The behavior of this material with respect to Ca uptake contrasts strikingly with that of excised barley roots. Moore et al. (4) have proven conclusively that in whole roots of barley seedlings uptake of Ca is almost, if not entirely, a non-metabolic process. In view of this difference in behavior between the two species, chemical determinations of Ca uptake were made to eliminate the possibility that the apparent uptake of Ca measured with Ca^{45} was the result of isotopic exchange. These determinations were carried out with the terminal 11.8 mm of the root tip in order to have sufficient material for accurate analysis. The initial Ca content was 9.63 meq per 1,000 g. After 24 hours in 0.005 N $CaCl_2$ at 26 C the Ca content was 60.7 meq. This value conforms well with that which may be calculated from the 24 hour uptake figures for the individual sections determined by measurements of radioactivity and confirms the supposition that Ca is metabolically absorbed by vacuolated tissue of maize roots. This finding suggests that maize root tips may prove ideal material for studies of salt induced respiration since by proper sectioning one can separate tissue which actively accumulates salt from that in which ion uptake is purely passive. It may thus be possible to separate respiratory stimulations resulting from the operation of the accumulation mechanism from those

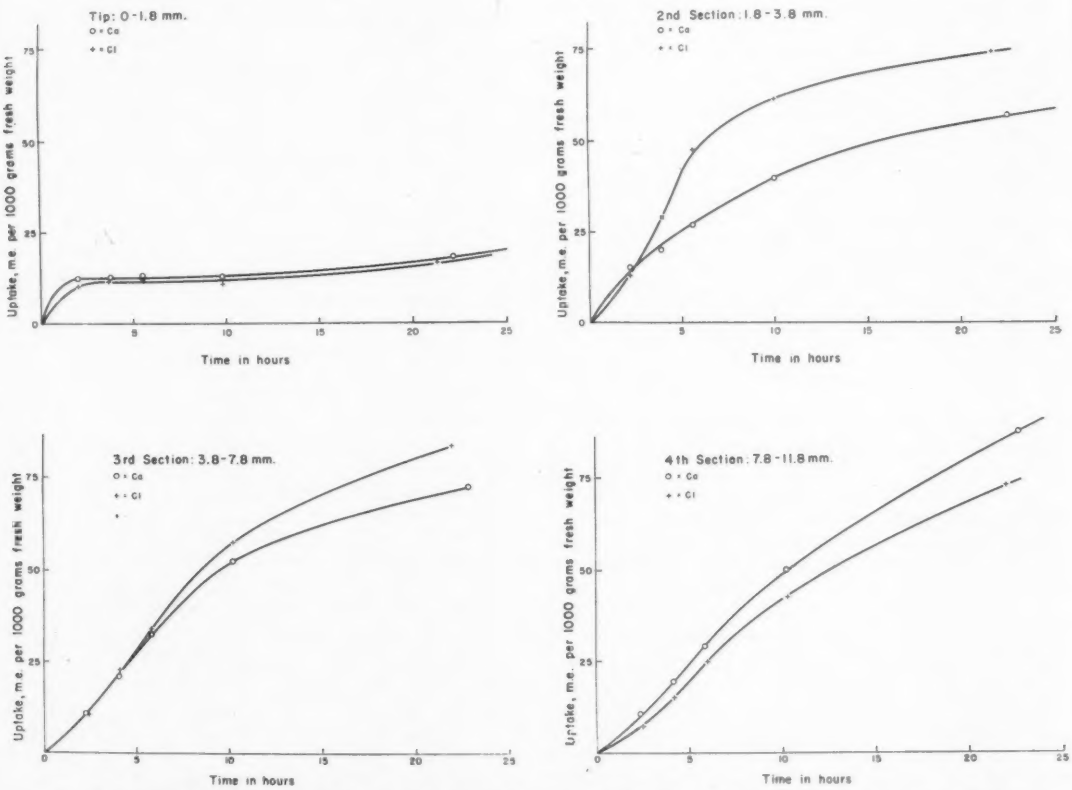

FIG. 2. Calcium and chloride uptake versus time for various sections of maize roots at 26 C. For each curve, 40 segments of tissue were placed in 500 ml of 0.005 N $CaCl_2$ labeled with 0.02 μc of Ca^{45} or Cl^{36} per ml. The pH of the experimental solutions was 6.0. At the intervals indicated in each curve, the segments were removed briefly from the medium, blotted dry, and counted. The uptake of calcium or chloride as meq/1000 g fresh weight was calculated from the known fresh weight of the segments.

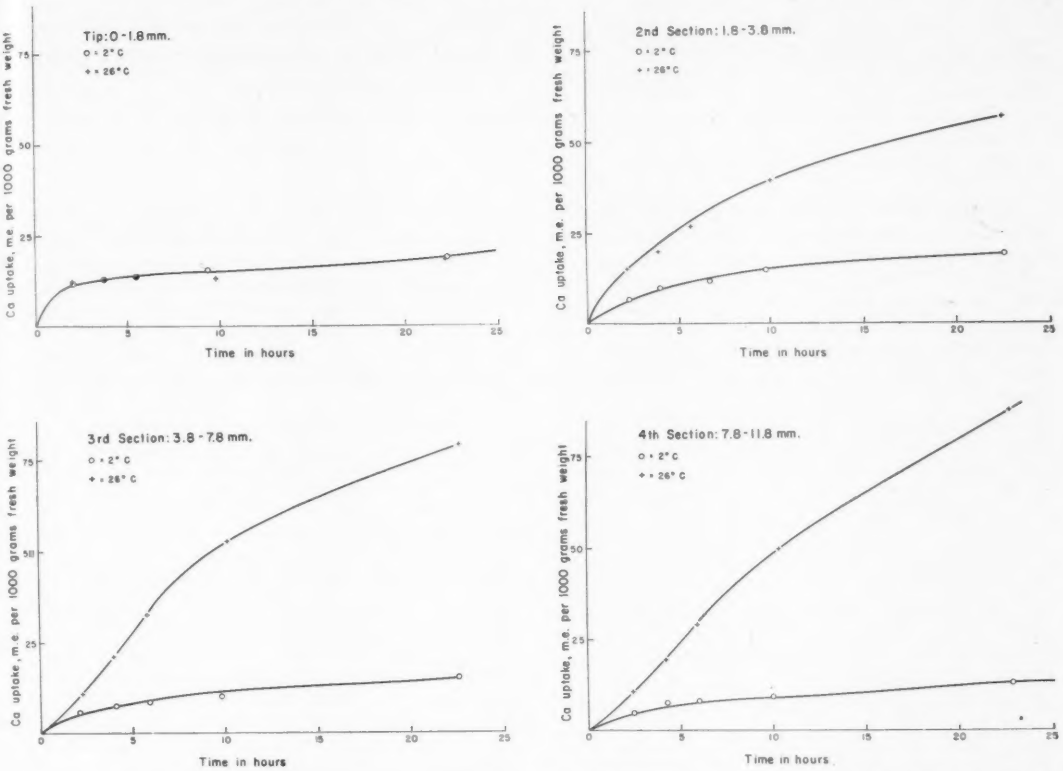
Tip: 0 - 1.8 mm.
o - 2° C
+ - 26° C
2nd Section: 1.8 - 3.8 mm.
3rd Section: 3.8 - 7.8 mm.
4th Section: 7.8 - 11.8 mm.
Ca uptake, m.e. per 1000 grams fresh weight
Time in hours

FIG. 3. Calcium uptake versus time curves for various sections of maize roots at 2 C and 26 C. For each curve, 40 segments of tissue were placed in 500 ml of 0.005 N $CaCl_2$ labeled with 0.02 μc of Ca^{45} per ml. The pH of the experimental solutions was 6.0. At the intervals indicated in each curve, the segments were removed briefly from the medium, blotted dry, and counted. The uptake of calcium as meq/1000 g fresh weight was then calculated from the known fresh weight of the segments.

due to other causes such as specific effects upon enzyme systems. This work, under way, will be reported shortly.

SUMMARY

The absorption characteristics of vacuolated and non-vacuolated sections of the primary root of *Zea mays* with respect to the uptake of Ca and Cl have been determined. As in the case of sodium uptake, the uptake of both Ca and Cl was a non-metabolic process in the meristematic portion of the root tip. The uptake of these ions into vacuolated portions of the root was strongly temperature dependent and, hence, largely metabolic.

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CHANGES IN NITROGEN METABOLISM IN SWEET POTATO WITH BLACK ROT[1,2]

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Although recent studies on changes in nitrogen metabolism in diseased plants have accumulated, their relationship to resistance or susceptibility is still ambiguous. Rohringer (21), and Shaw and Colotelo (24) reported differences in free amino acids between wheat (*Triticum aestivum*) infected by *Puccinia graminis* and the healthy plant. Rohringer, Stahmann, and Walker (23) found that following infection of tomato (*Lycopersicon esculentum*) leaves by *Fusarium oxysporum* f. lycopersici, amino acids, and other ninhydrin positive compounds were changed in concentration in the susceptible variety, in contrast to little change in the resistant one, though the change may not be a specific response to infection. Uritani (37) observed a decrease in asparagine in sweet potato roots (*Ipomea batatas*) infected by *Ceratocystis fimbriata*, and Suzuki et al. (31) reported the disappearance of asparagine, threonine, and serine in sweet potato roots infected by *Helicobasidium mompa*. Barnett and McLaughlin (4) reported that the electrophoretic pattern of the protein components of wheat leaves infected by *Puccinia triticina* was different from that of healthy leaves. Heitefuss, Buchanan-Davidson, Stahmann, and Walker (13) carried out electrophoretic and immunochemical analyses for protein extracts of resistant and susceptible cabbage (*Brassica oleracea*) seedlings, uninoculated and inoculated with *Fusarium oxysporum* f. conglutinans; the immunochemical results with rabbit antisera showed up to seven antigenic components in extract of infected susceptible cabbage, compared to four components in susceptible healthy, resistant healthy and resistant inoculated varieties. Akazawa and Uritani (3), Tomiyama et al. (33) and Suzuki et al. (31) found an apparent increase in protein nitrogen in sweet potato infected by *C. fimbriata*, in potato (*Solanum tuberosum*) infected by *Phytophthora infestans*, and in sweet potato infected by *H. mompa*, respectively. Furthermore, it was found by Akazawa and Uritani (1, 2) that in the healthy tissue of sweet potato adjacent to the infected tissue, protein increased in every cellular fraction (microsomes, mitochondria, & supernatant), and changes in the molecular structure of some proteins occurred as shown by a difference in their electrophoretic patterns. Our present study is an extension of the investigations on nitrogen metabolism in the diseased sweet potato.

MATERIALS & METHODS

Sunnyside and Orange Little were chosen as resistant and susceptible varieties of sweet potato [(*Ipomea batatas* L. (Lam.)], respectively. The roots of both the varieties were grown at the experimental farm of the U.S. Department of Agriculture, Beltsville, Md., under comparable conditions, and harvested in October of 1958 and 1959, cured, and sent to Madison without being chilled. The roots were stored at 11 to 13 C, without fungal damage or noticeable sprouting. Individual roots were cut into two parts lengthwise, both parts were sliced 3 cm thick, respectively. One group was inoculated with a spore suspension of *Ceratocystis fimbriata* (E. H.) Ell. and incubated at 38 C for 40 to 48 hours. The mycelium spread over the surface and also penetrated into the tissue. Sections (2–3 mm thick) from the non-infected region just below the infected part where the fungus was growing were taken to extract the nitrogen compounds. This tissue is called diseased tissue. The other group was incubated under the same conditions without inoculation, and sections (2–3 mm thick) were taken, from the tissue 0.5 mm below the surface, as a control for checking the effect of slicing. This tissue is called sliced tissue. Sometimes tissue of whole root was used without any inoculation and incubation. This tissue is called healthy tissue.

EXTRACTION OF SUPERNATANT PROTEINS FROM SWEET POTATO TISSUES: These tissues (50–150 g) were homogenized with 50 to 150 ml of cold 0.05 M phosphate buffer (pH 7.3–7.5) containing 12.5 % (6.95×10^{-1} M) glucose, 2×10^{-3} M magnesium chloride ($MgCl_2$) and 1 % (5.7×10^{-2} M) ascorbic acid in a Waring blendor for 3 minutes. During the homogenization, the pH was adjusted to 7.0 to 7.5 with 10 % potassium carbonate by using pH paper. The homogenate was filtered through four layers of cheese cloth, and the filtrate was dialyzed for 5 hours against 30 volumes of the same buffer through which nitrogen gas had been bubbled, with four buffer

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changes. Dialysis was carried out under nitrogen. The dialyzate was centrifuged at 8,000 × *g* for 20 minutes and the supernatant was centrifuged again at 105,400 × *g* for 90 to 120 minutes. The supernatant was concentrated in a cold room by ultrafiltration through cellophane tube in vacuo, with dialysis against the buffer having the same composition except only 0.5 % ascorbic acid. Usually it was concentrated to one-half to one-third the original volume. Sometimes the supernatant was left frozen at −15 C overnight, before the concentration. The concentrate was dialyzed against the same buffer as used for ultrafiltration, and then against 0.05 M phosphate buffer containing 12.5 % glucose and 0.05 % ascorbic acid. The dialyzate was stored frozen at −15 C overnight, and centrifuged at 8,000 × *g* for 20 minutes. The supernatant was stored at −15 C until used. The reasons for using glucose, $MgCl_2$, and ascorbic acid in the extraction medium, and for predialysis immediately after the homogenization will be described in the discussion.

Extraction of Proteins From Culture Media of C. Fimbriata, & From Mycellium & Spores A: Extraction from liquid culture medium—the pathogen was grown with shaking at 25 C for 3 days in potato extract with 2 % glucose, 0.2 % casein hydrolyzate and 10 μg % vitamin B_1. The final pH was 6.0 and pH was adjusted to 6.7. The medium was centrifuged, dialyzed against 0.05 M phosphate buffer (pH 7.5) containing 6 % glucose, concentrated by ultrafiltration, and the concentrate was dialyzed against the same buffer, and centrifuged. The culture medium without inoculation was treated in the same way, as a control. B: Extraction from wheat bran medium—5 g of wheat bran were mixed with 10 ml of distilled water in each Erlenmeyer flask, sterilized in an autoclave, inoculated with spores of the pathogen, and left at 28 C for 5 to 8 days. After the fungus had grown well, 50 ml of distilled water and 10 drops of toluene were then added to each flask, which was shaken gently overnight. The suspension was filtered through cheese cloth, and was centrifuged. The supernatant was saturated with ammonium sulphate, left at 4 C for several hours, and centrifuged at 4,500 rpm for 30 minutes. The precipitate was dissolved in a small volume of distilled water, then the solution was dialyzed against distilled water and centrifuged at 9,000 rpm for 20 minutes. C: Extraction from the mycelium and spores—by the centrifugation procedure described in A, the mycelium and spores were separated from the culture medium. Those were ground by freezing and thawing in a mortar, with 2 ml of 0.05 M phosphate buffer (pH 7.5) containing 6 % glucose. After adjustment to pH 7.0., the homogenate was filtered through cheese cloth, dialyzed against the same buffer, and the dialyzate was centrifuged.

Method of Immunization: We used the same immunization procedure as Heitefuss et al. (13) used with cabbage proteins. One part of the protein extract was emulsified with one part of adjuvant. Each protein preparation (20½ mg) in 6.0 to 10.0 ml of the emulsions was injected subcutaneously into rabbits. Three rabbits were immunized with each of the four extracts from diseased and sliced tissues of both varieties. The first bleeding was made 4 to 5 weeks after the injection. Immunochemical test showed that the antisera were weak, therefore, 5.5 weeks after the first injection, half the original amount in adjuvant was injected into the rabbits. The second bleeding was made 9 weeks after the initial injection. The antisera were frozen at −15 C.

Antigen-Antibody Test: The precipitation reaction of normal sera and antisera with antigens was investigated by the double diffusion method (16, 17) in agar gel in petri dishes. The protein samples injected for immunization and samples extracted later by the same procedure were used as antigens. The antisera and concentrates obtained by precipitating with one-third saturated ammonium sulphate (22) were used as antibody sources. Twenty milliliters of 1.2 % agar solution containing 0.9 % sodium chloride (NaCl) and 20 mg % merthiolate were poured into each petri dish. After solidifying, one hole at the center and six symmetrical holes at the distance of 1.8 to 2.0 cm from center were made, and filled with normal serum or antiserum, and antigens, respectively. Sometimes the reverse combination was used. Diameters of the holes for normal serum or antiserum, and for antigens were 8.3 and 6.0 mm, respectively. The dishes were kept in a moist chamber at 20 ± 1 C. Every day for 10 days, the precipitation patterns were observed. If a line of one antigen-antibody system forms a continuous arc with that of another system, the antigen is immunologically identical. But if it intersects or crosses, the antigen is not the same.

Immunochemical Absorption Test: To determine whether or not a particular antigenic component was contained in some samples, or to estimate how much it was present, the absorption method was used according to the quantitative serum-agar technique of Wright (44). One part of an antiserum (AS–A) toward a preparation A was mixed with one part of a serial dilution of preparation B and the mixtures were left in cold. After 2 days, preparation A was put into the central hole, and the mixtures of AS–A with preparation B, and AS–A itself were put into the six holes around the center. If a particular antigenic component is not contained or is present in a very small amount in preparation B, and the mixtures as well as AS–A still contain similar amounts of unreacted antibody, precipitation arcs will form between A and each of the outer holes forming a continuous ring of precipitate. If that antigenic component is present in B, the antibody in AS–A is absorbed by it, thus the specimen which absorbed all the antibody in a certain volume of AS–A cannot give a precipitation line in the agar gel. Quantitative estimation of the antigenic component in B is expressed as the range between the minimum amount of B absorbing all the antibody and the maxi-

mum amount of B which does not absorb all the antibody. Such amounts are expressed as the dilution values of the antigenic extract required to reach this range.

ELECTROPHORESIS IN STARCH GEL: For starch-gel electrophoresis, the methods of Smithies (25, 26, 27) were followed using both horizontal and vertical migration; in most experiments, the latter was used. Hydrolyzed starch (12.5–12.8 %, prepared by Connaught Med. Res. Lab., Toronto, Canada) was mixed with borate buffer (0.025 M boric acid & 0.01 M NaOH, pH 8.1 in the gel) and gently heated. Electrophoresis was carried out at 5.0 to 5.5 v per cm for 6 hours at 4 C. After electrophoresis the gel was cut in half horizontally so that each strip contained the complete series of proteins, and the protein patterns stained with saturated amido black in a solution of methanol: water: acetic acid (5: 5: 1), and washed in the same solution without dye. To obtain lipid protein staining, the gel was put into saturated sudan black in 60 % methanol and washed with 50 % methanol (9, 32).

STARCH-GEL IMMUNOELECTROPHORESIS: Immunoelectrophoresis in agar gel was described by Graber (9); recently Poulik (26) developed a method in which immunochemical reaction was combined with electrophoresis in starch gel. In principle the same method was applied here. Each sample was subjected to electrophoresis in starch gel. After electrophoresis, the gel was cut in half horizontally, and sections 6 mm wide were cut longitudinally and at a right angle, from the lower half of the starch gel which contained the proteins separated by electrophoresis, and placed on a glass plate. An agar solution at 40 C was poured into the plate, to the same thickness as that of the sections. Spaces for inserting antisera were made outside the antigen sections with glass rods 7 mm wide, which were removed after solidification. The distance between starch-gel sections and antiserum reservoirs was 8 mm. After filling the reservoirs with antisera, the plate was left in a moist chamber.

In order to determine which pattern on immunoelectrophoresis corresponded to each of the amido black-stained patterns on starch gel, and to each of the immunochemical patterns on agar gel in petri dishes, the starch-gel section was cut into pieces (3–5 mm). The pieces were placed in a circle in petri dishes and the agar solution at 40 C was poured around them. After the agar had solidified, the antiserum was put into a hole at the center of the circle. The original tissue extract was often put adjacent to the cut blocks in the same circle; after that, precipitation lines were obtained.

TEST OF POLYPHENOL OXIDASE & PEROXIDASE ON STARCH GEL: Owen et al. (18) investigated peroxidase activity on starch gel after electrophoresis. We also investigated oxidizing enzymes on starch gel. Polyphenol oxidase on starch gel after electrophoresis was detected as follows: A piece of filter paper wet with 0.5 % chlorogenic acid solution (pH 6.7) was placed on the starch gel for 15 minutes at 28 C. Another filter paper wet with 1 % alanine solution (pH 6.7) was placed over the first filter paper, and the gel was left again for 25 minutes at 28 C. The enzyme was detected by a brown color of the reaction product of alanine with the quinone produced from chlorogenic acid by polyphenol oxidase. The reaction was stopped by placing the gel in 10 % acetic acid. Peroxidase was located as follows: The gel was dipped into 1 % pyrogallol solution for 1 minute. After draining, 1 % hydrogen peroxide solution was sprayed on the surface of the gel. A yellow brown band of purpurogallin appeared on the position of peroxidase. After 5 minutes, the gel was dipped into 10 % acetic acid to stop the reaction. Oxidation of pyrogallol by polyphenol oxidase was not appreciable within this time.

The test of peroxidase was applied also to determine if peroxidase was present among the immunochemical precipitation lines in agar gel. The procedure of Uriel (36) was used for this experiment. After obtaining precipitation lines between tissue extracts and antisera in agar gel, the gel was washed with 0.9 % NaCl solution containing 10 mg % merthiolate for 2 to 3 days in several changes of the washing solution. Then we investigated the peroxidase activity in the immunochemical pattern.

CONCENTRATION OF A PARTICULAR PROTEIN IN DISEASED TISSUE BY MODIFIED CELLULOSE CHROMATOGRAPHY: Tomiyama in unpublished work from this laboratory showed several protein patterns in white potato extracts by modified cellulose chromatography; the method he used was followed for the chromatographic study of the proteins in diseased tissue extracts. An extract from diseased tissue of Sunyside was dialyzed against 0.01 M tris-phosphate buffer (pH 7.96) containing 12.5 % glucose. Five milliliters (5.8 mg N) were put onto a DEAE-cellulose (100–200 mesh, 0.8 meq/g) column, and fractionated by gradient elution using 400 ml of the starting buffer (0.01 M tris-phosphate buffer, pH 7.96) and 200 ml of the limit buffer (0.1 M NaH_2PO_4 & 1 M NaCl). The optical density of each 5 ml eluent was measured at 260, 280, and 400 mμ. After that three to five fractions were combined, dialyzed against 0.05 M phosphate buffer (pH 7.5) containing 5 % glucose and 5 mg % merthiolate, and concentrated by ultrafiltration to about 0.3 ml. The concentrates were subjected to immunochemical test, electrophoresis, and nitrogen determination.

ULTRACENTRIFUGAL ANALYSES OF PROTEIN FRACTIONS: Several sweet potato roots were cut in half lengthwise, one half was sliced, inoculated with spores of the pathogen, and incubated at 28 C. The other half was kept at 20 C without slicing. Tissue (40 g) was collected from each of the healthy tissues adjacent to the infected one, and of the healthy, uninoculated tissue, and homogenized in a mortar with sea sand and 40 ml of 0.05 M phosphate buffer (pH 7.5) containing 2×10^{-3} M $MgCl_2$ and 1 % ascorbic acid.

The homogenate was filtered through cheese cloth, and centrifuged at 8,000 × *g* for 20 minutes. The supernatant was centrifuged at 105,000 × *g* for 120 minutes. The precipitate was suspended in the same buffer, and made up to 3 ml. The centrifugal patterns in the suspension were studied in the analytical ultracentrifuge at 35,600 rpm, and 47,660 rpm.

EXTRACTION OF FREE AMINO ACIDS & THE ANALYSES: Inoculation of the pathogen on the tissues, and sampling were almost the same as shown above for extraction of supernatant proteins. Each of four roots was cut and divided into three groups, healthy, sliced, and diseased tissue. From each group 20 g of tissue were taken for extraction by a modification of Rohringer's method (21). Each sample was heated with 200 ml of 70 % acetone for 2 hours at 60 C, to destroy enzymes such as polyphenol oxidase. The mixture was homogenized in a Waring blendor for 2 minutes, transferred to the flask with 50 ml of 50 % acetone, heated for 1 hour at 60 C, and filtered by suction. The residue was suspended in 200 ml of 50 % acetone, heated for 1.5 hours at 60 C, and filtered. This was repeated again, and the last residue was washed with 200 ml of 50 % acetone and filtered. These filtrates were combined and concentrated in vacuo. The concentrate was deproteinized by mixing with an equal volume of 1 % picric acid solution (29), and centrifuged. The supernatant was passed through a column of Dowex 2 to remove picric acid. This procedure also removes polyphenols which may oxidize and react with amino acids. The eluent was concentrated in vacuo to a concentration appropriate for the analysis with a Spinco amino acid analyzer (28).

DETERMINATION OF INSOLUBLE & SOLUBLE NITROGEN: The samples were mixed with trichloroacetic acid solution, and divided into insoluble and soluble fractions, which were digested with sulfuric acid and assayed for nitrogen with Nessler's reagent.

QUALITATIVE TEST ON TISSUE FOR IPOMEAMARONE: Ipomeamarone and similar compounds react with Ehrlich's reagent (10 % *p*-dimethylaminobenzaldehyde in 40 vol. % sulfuric acid) to produce a pink color (41). When Ehrlich's reagent is applied to the tissue, that part containing ipomeamarone develops a pink color.

EXPERIMENTAL RESULTS

CHANGES IN TISSUES IN RESPONSE TO INFECTION: It was found by Cheo (7) and Hildebrand (14), that varieties Sunnyside and Orange Little were respectively resistant and susceptible to *C. fimbriata*. Though growth of the aerial hyphae on the tissue surfaces was almost the same in both varieties 2 days after the infection, Sunnyside showed a clear resistant action, as strong browning in the infected area, 0.2 mm thick, and production of ipomeamarone only in that area, in contrast to Orange Little which showed light browning in the infected area, 0.5 mm thick, and production of the compound both in the infected and adjacent tissues (38, 39, 41). Penetration of the pathogen was stopped completely within 3 days in the case of Sunnyside, but in the case of Orange Little, penetration was not stopped, though some resistant action appeared and the rate of the penetration decreased after 3 to 5 days.

Varieties Norin No. 1 or No. 10, and Norin No. 4 or No. 5 were used as the resistant and susceptible varieties, respectively, for experiments in Japan. The above mentioned observations were almost the same as those seen in Norin No. 1 or No. 10 and No. 4 or No. 5 (38, 39). Hence we may relate the results of these experiments to those in Japan.

NITROGEN CONTENT OF TISSUE & EXTRACTS: In table I is presented the nitrogen content of the tissues and of the protein extracts. In response to the infection, protein increased about 10 to 30 % in diseased tissue (3), and proteins of supernatant, mitochondrial, and microsomal fractions also increased (1). In these experiments the increase in protein of diseased tissue was also seen. But the amount of protein in the extracts was less in diseased tissue than in sliced tissue in most cases. The above results may be explained by precipitation of smaller amounts of protein extracted from diseased tissue, during extraction procedures such as pre-dialysis after the homogenization and concentration by ultrafiltration.

IMMUNOCHEMICAL REACTION IN AGAR GEL: Tests for a non-specific reaction of normal sera with the healthy, sliced, and diseased tissue extracts showed that normal sera caused slight turbidity after reaction with the tissue extracts in ring tests, but neither precipitation nor turbidity was shown on diffusion through agar gel. Therefore, all precipitation lines in agar between antisera and tissue extracts are due to a specific antigen-antibody reaction.

The precipitation reactions of concentrated antisera toward extracts from healthy, sliced, and diseased tissue extracts were studied. In the case of the resistant Sunnyside shown in figure 1, I and II, the reaction of diseased tissue extracts (D_1 & D_2) with their own antisera (AS_D) indicated two to five distinct antigens which were not present or were detected only in very small amounts in healthy (H) and sliced tissue (S_1 & S_2) extracts. Two showed intense lines which are indicated as B and D in figure 1–II, and are referred to as B_s or D_s to indicate B and D from the Sunnyside variety. The line B_s was more clear than D_s. The numbers of additional lines depended on the combination of the three antisera toward diseased tissue extract and the three diseased tissue extracts. These distinct additional lines were not obtained from antisera toward sliced tissue extract when reacted with healthy, sliced, or diseased tissue extracts. Two other components, A and C in figure 1–II, are called A_s and C_s to indicate from Sunnyside, and were found commonly in all combinations between antisera and extracts. One new component was produced in response to slicing. This

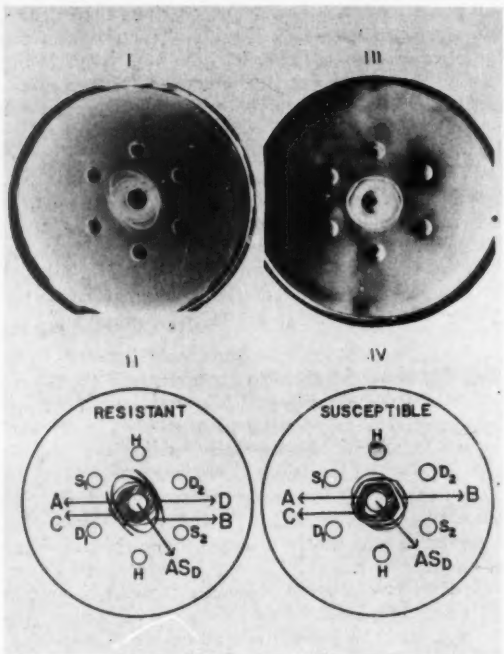

FIG. 1. Precipitation reactions of antisera with protein extracts in agar gel. (I, II) Sunnyside (resistant variety); (III, IV) Orange Little (Susceptible variety); H = healthy tissue extract; S_1 S_2 = sliced tissue extracts I and II; D_1, D_2 = diseased tissue extracts I and II; AS_D = concentrated antiserum toward diseased tissue extract I; A, B, C, D = components shown as precipitation lines.

was contained also in diseased tissue extracts, but very little or none at all was seen in healthy tissue extract. When diseased tissue extracts of the susceptible Orange Little were reacted with their own antisera as shown in figure 1, III and IV, there was a faint line from one component B, (or B_o to indicate B from Orange Little) which was not found in the reaction with healthy or sliced tissue extracts. Two other components, A_o and C_o (indicated as A & C in fig 1–IV), were detected in every extract. No distinct component was found from sliced tissue extracts when reacted with their own antisera.

Since distinct antigenic components were found in all extracts from diseased tissues of roots harvested in 1958 and 1959, it seemed that they represented components appearing in the host itself in response to the infection. But, there was a slight possibility that native proteins in diseased tissue were changed or denaturated during extraction in the presence of abnormal metabolites such as polyphenols, and the denaturated proteins caused the antibody formation. To test this point, protein extracts were prepared from diseased and healthy tissues of Sunnyside, by two other methods.

A: Homogenates after the grinding: 5 g of each tissue were ground in a mortar with 5 ml of cold 0.05 M phosphate buffer (pH 7.5) containing 5 % glucose, 1.5 % ascorbic acid, and 0.02 % merthiolate. The homogenates were filtered through cheese cloth, and put into the holes in agar-gel dishes. After adding antiserum, the dishes were stored under nitrogen. B: Acetone powder extracts: 10 g of each tissue were homogenized with 0.02 % ascorbic acid-containing acetone at −30 C, and acetone powder was prepared. It was homogenized with 9 ml of the same buffer as shown in A, and the immunochemical reactions were investigated as shown above using the extracts.

In both cases, distinct patterns appeared. In diseased tissue extracts patterns were seen after 1 to 2 days, and the largest ones formed continuous arcs with the main patterns shown as B and D in diseased tissue extract. This is evidence that the distinct components are produced by diseased tissue and not in the isolation of their proteins.

Since diseased tissue extracts were prepared from healthy tissue adjacent to the infected area, it was unlikely that such specific patterns were the result of immunization by proteins of the fungus or those

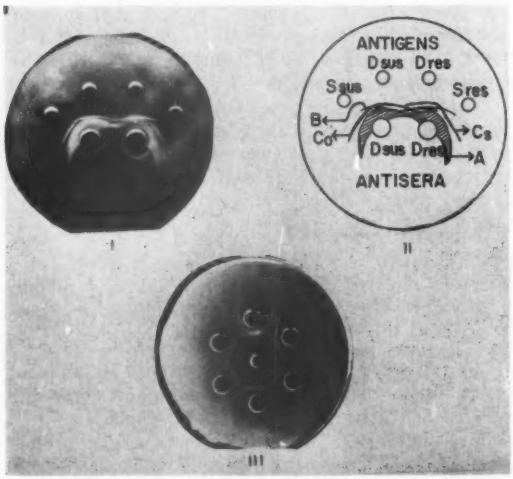

FIG. 2. Immunochemical comparison of the antigens from Sunnyside with those from Orange Little, and of the diseased tissue antigens with those from the sliced tissue. I, II: Comparison of immunochemical reactions of diseased tissue extracts of Sunnyside with those of Orange Little; ANTIGENS, Dres, Sres, Dsus, Ssus = diseased (D) and sliced (S) tissue extracts of Sunnyside (res) and Orange Little (sus); ANTISERA, Dres, Dsus = concentrated antisera toward diseased (D) tissue extracts of Sunnyside (res) and Orange Little (sus). III: Absorption of antiserum toward diseased tissue extract of Sunnyside by sliced tissue extract of Sunnyside. The center hole contained diseased tissue extract, and the hole just above the center one was filled with antiserum toward that extract. The five others contained mixtures of that antiserum and sliced tissue extract in which the volume of the antiserum to that of the extract were as follows: 10, 5, 2.5, 1.25 and 1, clockwise.

excreted by it. To confirm this point, protein extracts of the pathogen and of the cultural media were tested. They gave no reaction with any of the antisera studied or with extracts of healthy, sliced, or diseased tissue.

IMMUNOCHEMICAL COMPARISON OF SUNNYSIDE WITH ORANGE LITTLE, & OF DISEASED TISSUE WITH HEALTHY & SLICED TISSUES: To compare the antigenic components in diseased tissues of resistant and susceptible varieties the experiments illustrated in figure 2 were done. Figure 2–I, II, shows that the line from component B_s of Sunnyside (Dres) forms a continuous arc with that from component B_o of Orange Little (Dsus). A continuous arc was also formed between diseased tissue extracts of Sunnyside and the extracts of Orange Little. From those results B_s and B_o were shown to be immunochemically identical, and so that component is now called component B. Absorption results (table I) showed the amount of B in the susceptible Orange Little, less than half that in the resistant Sunnyside, greatly increases in both varieties on infection.

It was shown by the immunochemical absorption experiments illustrated in figure 2–III that the antibody toward component B in diseased tissue extracts of Sunnyside was not completely absorbed by sliced or healthy tissue extracts of Sunnyside or Orange Little. Therefore, it was clear that some antigens were produced by the host tissue in a great amount in response to the infection, and were present in a small amount in healthy and sliced tissues (fig 2–III, table I), or that these precipitation lines from diseased tissues of Sunnyside contained components not present in the extracts of healthy or sliced tissues.

Components A_s and A_o were identical, since they formed a continuous arc (fig 2); they are called component A. It was contained in every extract of both varieties, but less in the tissues of Sunnyside than those of Orange Little. It was decreased in response to the infection (table I).

It was shown by the data in figure 2 that component C_s was not the same as component C_o, for these precipitation lines clearly do not merge into one but cross over. Absorption results indicate that C_o was also contained in diseased tissue of Sunnyside, but in a less amount than that of Orange Little, and was present in the same amount in diseased and sliced tissues of Orange Little (table I).

EXPERIMENTS WITH JAPANESE VARIETIES: Subsequent experiments carried out in Japan with Japanese varieties of sweet potatoes have given similar results to those obtained in Madison. They indicate that the same antigens, B and D, were produced in all four Japanese varieties in response to infection.

TABLE I

NITROGEN CONTENT OF TISSUES & EXTRACTS, & RELATIVE AMOUNTS OF ANTIGENS IN EXTRACTS

	SUNNYSIDE			ORANGE LITTLE		
	H[a]	S[a]	D[a]	H[a]	S[a]	D[a]
Whole roots						
Insol. N[b]	1,031			1,080		1,218
Sol. N[b]	1,203			688		361
Extracts I[c]						
Insol. N		726	656		1,095	875
Sol. N		48	98		64	94
Antigen A[d]		10	4–10		> 10	10
" B[d]		< 1	10–20		< 1	2–4
" C_o[d]			1–1.3		2–4	2–4
Extracts II[e]						
Insol. N	1,276	1,810	1,162	850	1,380	1,148
Sol. N	74	70	86	84	100	92
Antigen A	Healthy =	Sliced	Diseased[g]	Healthy =	Sliced	Diseased[g]
" B	< 1	< 1	20	< 1	< 1	4–10
Extracts III[f]						
Insol. N		2,164	1,932		2,640	2,926
Sol. N		85	68		88	131
Antigen A		Sliced	Diseased[g]		Sliced	Diseased[g]
" B		< 1	10–20		< 1	4–10

[a] H, S, D: Healthy (H), sliced (S), and diseased (D) tissues, respectively.
[b] Insol. N and sol. N express γ of nitrogen precipitated and not precipitated with trichloracetic acid solution in 1 g of fresh tissues or 1 ml of extracts.
[c] Prepared at the end of May, 1959, and injected into rabbits.
[d] Antigens A, B, and C_o: Relative amounts of components showing precipitation lines in immunochemical tests.
[e] Healthy samples were prepared in the middle of July, 1959; sliced and diseased samples at the beginning of August, 1959.
[f] Prepared at the beginning of November, 1959.
[g] Qualitative relationship was shown.

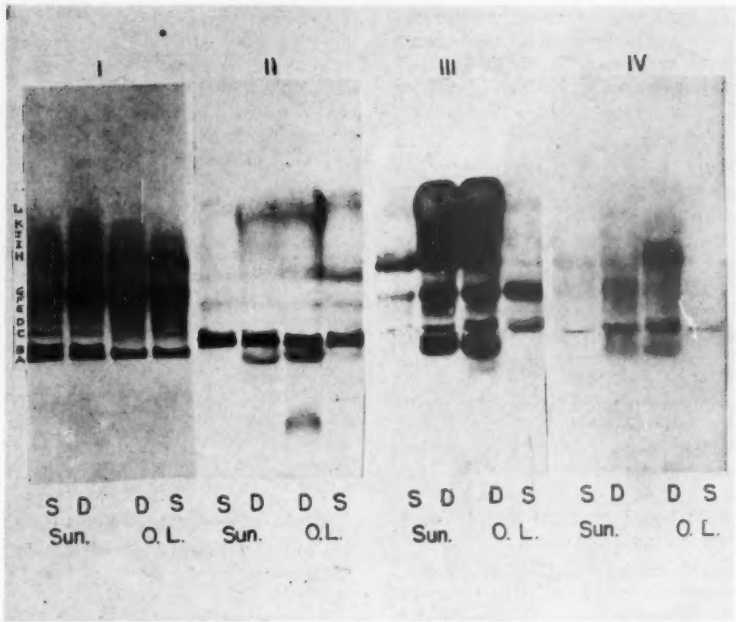

FIG. 3. Starch-gel electrophoresis of sliced and diseased tissue extracts. I: Protein patterns stained by amido black; II: patterns stained by sudan black; III: patterns of polyphenol oxidase; IV: patterns of peroxidase; S = sliced tissue extract; D = diseased tissue extract; Sun. = Sunnyside; O.L. = Orange Little.

The amounts of these two components were much larger in the diseased tissue. The amount of antigen B in diseased tissue was larger in the highly resistant variety No. 10 than in the less resistant No. 1, which was also greater than that in the susceptible varieties No. 4 and 5. The amount of antigen D in diseased tissue was larger in the resistant No. 10 variety than in the other less resistant or susceptible varieties. Both antigens B and D were produced much more in diseased tissue which was left for 2 days after the inoculation than in that left for only 1 day. These experiments with Japanese varieties confirm those made in Madison and indicate that resistance in the Japanese varieties as in the American varieties is correlated with the ability of the host tissue to increase its amount of antigens B and D in response to infection by the fungus (42).

STARCH-GEL ELECTROPHORESIS: Samples from healthy and sliced Sunnyside tissues showed the same electrophoretic patterns composed of 12 components (marked a, b,—k, l in fig 3–I). The same number of components were separated also from diseased tissue extracts, but the positions of the f and g components were different from those of healthy and sliced extracts, and the intensity of the e component of the former was less than that of the latter. All samples from healthy, sliced, and diseased Orange Little tissues showed the same patterns, and in place of f and g components which were present in Sunnyside, only one component was seen. The pattern of diseased tissue extracts was less intense than that from healthy or sliced tissue.

COMBINATION OF STARCH-GEL ELECTROPHORESIS WITH IMMUNOCHEMICAL REACTION: The results of the starch-gel immunoelectrophoresis experiments showed the protein patterns in more detail, and clarified the interrelationship between the agar-gel immunochemical patterns obtained in petri dishes and the stained patterns of starch-gel electrophoresis (fig 4–I, II, III).

Sunnyside: A: Component A seen in agar-gel petri dishes (fig 1) was divided into subcomponents A_1, A_2, and A′ and A_1, A_2, A′, and A″, respectively, in sliced and diseased tissue extracts. A_1 and A_2 seemed to be the main common components contained in every extract. A′ was present in diseased tissue in a much higher concentration than in sliced tissue, and A″ was seen only in diseased tissue extract. A′ and A″ showed cross reaction with A_1. B: Component B, a distinct component in diseased tissue, seen in the patterns obtained in the agar-gel in petri dishes, gave also a specific pattern on immunoelectrophoresis. It did not show a cross reaction and was not seen in sliced tissue extracts. It was located at the same positions as that of A″. C: A′, and A″ and B were located at the same position as those of e and f amido black stained patterns of starch-gel electrophoresis, respectively. D: The location of

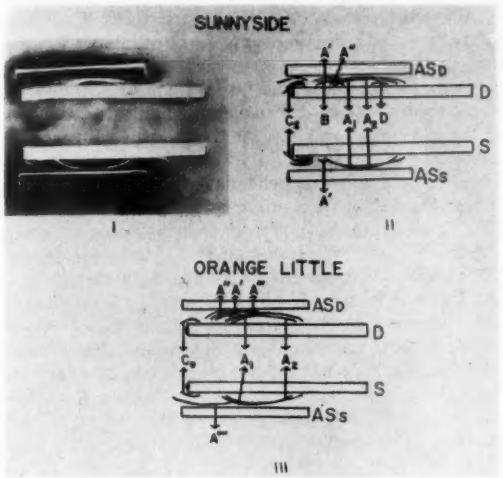

FIG. 4. Starch-gel immunoelectrophoresis. I, II: Sunnyside; III: Orange Little; D, S = sections of starch gel containing diseased (D) and sliced (S) tissue proteins separated by electrophoresis. AS_d, AS_s = Antisera toward diseased and sliced tissue extracts, respectively; A_1, A_2, A′, A″, A‴, A⁗, B, C_s, C_o, D = antigenic components showing specific precipitation lines.

A_1 and A_2 and component C_s corresponded to the h and b patterns on starch gel. E: There was a possibility that A′ and A″ might have been produced during extraction, and were not products of the diseased tissues. But the results of immunoelectrophoresis of the homogenates obtained by grinding and acetone powder extractions as above described showed that these components were also products of the diseased tissue itself. F: Component D_s in diseased tissue extracts, showed a distinct pattern in agar-gel diffusion and a faint line on immunoelectrophoresis located at the same position as for k pattern.

Orange Little: A: In diseased tissue extracts, three new components (A′, A″, A‴) were found which showed cross reaction with subcomponents A_1 and A_2 of component A. B: Component B in diseased tissue extracts of Sunnyside was not found in those of Orange Little after immunoelectrophoresis. Perhaps the amount was not enough to show the reaction. C: A′ and A″ were located at the position of the f pattern on starch gel, but were different from components A′ and A″ in the diseased tissue of Sunnyside, because the location of f pattern on starch gel was different between both varieties. The location of A‴ on the starch gel was not clear, because it showed a faint line. D: A_1 and A_2 and component C_o were located at h and b patterns on starch gel, in case of diseased and sliced tissues, as shown in Sunnyside. But as shown above (fig 2), C_s and C_o should be different between both the varieties. E: One component, A⁗, which cross reacted with A_1, found in sliced tissue extracts, was located at c pattern on starch gel. Therefore, A⁗ is different from A′, A″, and A‴ of diseased tissue extracts.

CHANGES IN PROTEIN PATTERNS BY OTHER STAINING & COLORING TECHNIQUES: The activity of polyphenol oxidase and peroxidase increased in sweet potato tissue in response to the infection (39, 40); two peroxidases were found in healthy sweet potato roots by Kondo and Morita (15). We experimented to see if the electrophoretic patterns of these oxidative enzymes on starch gel were changed in response to the infection. Also, we studied the pattern of lipoproteins on starch gel, since it was assumed that lipid metabolism should be accelerated by the infection.

Amido black staining for total protein showed little difference between diseased and sliced tissue proteins, but coloring by oxidative enzyme action and by the lipid staining sudan black showed drastic changes in diseased tissues of both varieties (fig 3). Figure 3–IV shows that peroxidase activity greatly increases on infection; there was weak peroxidase activity in three or four bands in both sliced tissue extracts but strong peroxidase staining in more bands after infection. Polyphenol oxidase activity showed a similar increase as a result of infection in both varieties. It was interesting that the enzymatic stains showed migration of some proteins to the negative electrode, which was not detected by amido black staining. Sudan black stained patterns, figure 3–II, were similar to those of peroxidase. Peroxidase might have oxidized some leuco form in the sudan black to give a blue to brown color in addition to staining of lipoproteins. All the figures of the starch-gel electrophoresis patterns can be compared directly, since the conditions of electrophoresis and photographing were held constant.

IDENTIFICATION OF COMPONENT D_s AS A PEROXIDASE: Component D, (fig 1) in diseased tissue extracts of the resistant Sunnyside provided a distinct immunochemical pattern which showed peroxidase action in the agar-gel patterns in petri dishes. There was no visible component comparable to D in diseased tissue extracts of the susceptible Orange Little, but the peroxidase test of the gel patterns showed a faint peroxidase line was present in the precipitation area of component A. This precipitation line could not be detected without that test. No peroxidase line was found in sliced tissue extracts of either variety in the gel-diffusion patterns. The peroxidase test after immunoelectrophoresis verified that d which was located at the position of the K-line by amido black staining was indeed a peroxidase. After immunoelectrophoresis, a peroxidase was found also in the diseased tissue extract of Orange Little at the same position as that of the k-line though no precipitation line was visible before the peroxidase test. The precipitation lines of components A_1 and A_2 might have masked that line (fig 4–III). From now on this peroxidase is called component D, common to both varieties.

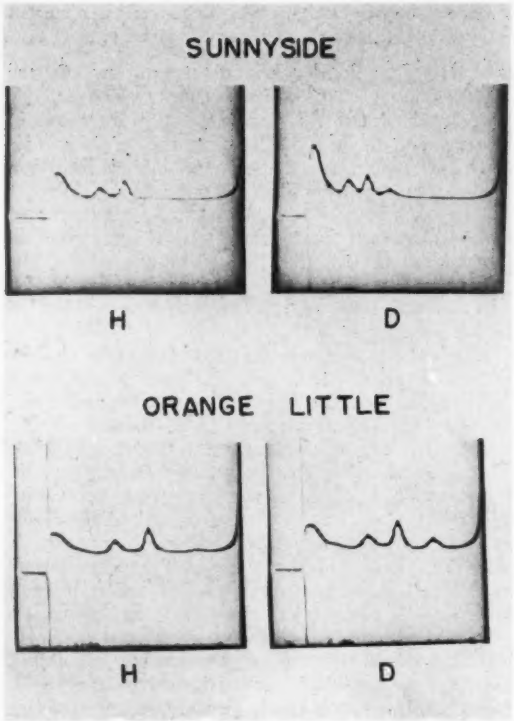

FIG. 5. Ultracentrifuge patterns of particles from healthy and diseased tissues. Speed 36,500 rpm. H = healthy tissue fractions; D = diseased tissue fractions.

On the other hand, the position of the k pattern corresponded to that of the peroxidase pattern showing the strongest action on starch gel after electrophoresis of diseased tissue extracts of both varieties (fig 3–IV). From the results of immunochemical and peroxidase tests on starch gel, it was verified that component D is contained in a larger amount in the diseased tissues of both varieties than in the sliced tissues. However, the extent of the difference between both varieties was not clear, but from the agar gel experiments it appears to be much higher in the resistant variety.

CONCENTRATION OF COMPONENT B BY CHROMATOGRAPHY: Chromatography on cellulose ion-exchange columns was used to concentrate component B from the diseased tissue extract of Sunnyside. From the results of 280 mμ absorption measurements of the eluents it was shown that the proteins in the extract were separated into several groups. It was found from the immunochemical test that component B was located in fractions from tube 11 to 28, especially from 11 to 16, and contaminated only by component A′. On concentration and electrophoresis of the contents of tubes No. 11 to 13 and of 14 to 16, a pattern was obtained which showed intense amido black staining at the position f in figure 3–I where B should be located. When determined by the immunochemical absorption test, the specific amount of B in the mixture from tubes 11 to 28 was 95 to 238 per mg protein in comparison with 2.7 in the original extract. Therefore, the component was concentrated 35- to 87-fold.

ULTRACENTRIFUGAL ANALYSES OF PROTEIN FRACTIONS: In most cases, there were three patterns composed of 73 S, 56 S, and 38 S units (fig 5). A group having a slow sedimentation coefficient (about 4 S) was present, but the members of this group were not separated from each other though centrifuged at 47,660 rpm for 1 hour. The three components can be assumed to be microsome particles from their sedimentation coefficients. Nucleic acid analysis by the method of Basler and Commoner (5) showed larger amounts of nucleic acid in comparison with those of the supernatant of low speed centrifugation (8,000 *g* for 20 min). More of the 73 S unit was in diseased tissues than in healthy tissues. By measuring the area bounded by the solute peaks and the solvent baseline, an estimation of the relative amounts of the components in each tissue preparation was obtained, using the assumption that each component has almost the same specific refractive increment. In both varieties, the total relative amounts were larger in diseased tissue than in healthy (table II). This agrees with the report of Akazawa (1) in which microsome protein increases together with proteins in other fractions, in diseased tissue.

CHANGES IN FREE AMINO ACIDS: In both varieties, the amounts of some free amino acids were changed in the diseased tissue extract, in comparison

TABLE II

RELATIVE AMOUNTS OF COMPONENTS DETERMINED IN ANALYTICAL ULTRACENTRIFUGE

VARIETIES	HEALTHY TISSUE				DISEASED TISSUE				RATIOS[a]
	38 S	56 S	73 S	Total	38 S	56 S	73 S	Total	
Orange Little	409	836	93	1338	478	905	316	1699	1.27
Sunnyside	280	476	...[b]	756	303	469	193	965	1.28

[a] Ratios of total amounts of diseased tissue components to those of healthy tissue.
[b] Area too small to measure.

with the sliced or healthy tissue extract. In diseased tissues, leucine, isoleucine, proline, valine, tyrosine, and lysine were increased with a concomitant decrease of the amides and alanine (table III). Asparagine could not be separated from glutamine by the amino acid analyzer, but from paper chromatographic analyses asparagine changed more than glutamine. That coincided with the fact that asparagine was isolated in large amounts from healthy tissue of sweet potato, but was not isolated at all from diseased tissue (37).

That free amino acids were higher in Sunnyside than in Orange Little can be explained by the larger amount of amides in Sunnyside.

An unknown basic component was eluted just before ammonia in the analysis of every extract of both varieties. From its position, this component was assumed to be ethanolamine (28). It showed little change in response to the infection as did ammonia (table III).

Discussion

At first the method used to extract the supernatant proteins will be discussed on four points. A: The beneficial effect of polyhydroxy compounds such as glucose on the stabilization of plant proteins was pointed out by Heitefuss, Buchanan-Davidson, and Stahmann (12). Also in our experiments glucose was present during the extraction and storage of the proteins. Glucose functions not only to prevent the breakage of cell particles such as mitochondria and chromoplasts by maintaining a suitable osmotic pressure, (thus yielding supernatant protein fractions with minimum contamination from the other proteins), but, also, to retard precipitation and denaturation of proteins during prolonged storage. B: Several workers found that microsomes from plant tissues (35), as well as from animal tissues (11) and microorganisms (6) can be kept from disassociating by the presence of $MgCl_2$. This was true also in the case of sweet potato tissue. Ultracentrifugal analysis of the microsome fraction showed three classes of particles having sedimentation coefficients from 30 to 80 S in extracts containing $MgCl_2$ (2×10^{-3} M) that were not present in extracts without $MgCl_2$. Therefore, $MgCl_2$ was used as one component of the extraction medium to obtain supernatant proteins not contaminated by microsomal fragments. This component was removed from the concentrated extracts

Table III

Free Amino Acids in Tissues, & Ratios of Amount in Diseased Tissues to Those in Sliced & Healthy Tissue

Amino acid	μmoles of free amino acids/g fr tissue					Ratios		
	Sun.H[a]	Sun.S[a]	Sun.D[a]	O.L.S.[a]	O.L.D.[a]	Sun.D / Sun.S	Sun.D / Sun.H	O.L:D. / O.L.S.
Neutral & basic								
Aspartic acid	4.55	2.60	2.87	3.98	4.14	1.1	0.63	0.93
Threonine	1.52	1.16	1.05	0.69		0.96	0.69	
Serine	3.17	1.76	1.83	1.81	1.60	1.04	0.58	0.89
Asparagine + glutamine	24.80	19.1	12.3	1.42	0.62	0.64	0.50	0.44
Glutamic acid	1.81	2.55	1.82	2.04	2.20	0.71	1.00	1.08
Proline	0.23	0.13	0.32	0.23	0.39	2.46	1.39	1.70
Glycine	0.83	0.63	0.53	0.27	0.27	0.84	0.64	1.0
Alanine	4.90	1.40	0.79	0.66	0.47	0.56	0.16	0.71
Valine	0.79	0.32	0.43	0.61	0.87	1.34	0.54	1.42
Methionine	0.22	0.06	0.08	0.07	0.07	1.33	0.36	1.0
Isoleucine	0.20	0.12	0.36	0.27	0.66	3.0	1.80	2.44
Leucine	0.23	0.16	0.39	0.22	0.58	3.36	1.70	2.64
Tyrosine	0.35	0.12	0.26	0.075	0.37	2.17	0.74	4.93
Phenylalanine	0.97	0.59	0.71	0.44	0.48	1.20	0.73	1.09
Basic								
Hydroxylysine	0.044	0.015	0.032	0.007	0.004	2.14	0.73	0.57
Tryptophan	0.23	0.151	0.153	0.086	0.13	1.01	0.67	1.51
Lysine[b]	0.082	0.096	0.234	0.10	0.15	2.44	2.86	1.50
Histidine	0.88	0.72	0.86	0.125	0.14	1.19	0.98	1.12
Unknown[c]	0.48	0.68	0.78	0.79	0.62	1.15	1.62	0.79
Ammonia	1.02	1.25	1.58	1.41	0.88	1.26	1.56	0.62
Arginine	0.66	0.41	0.49	0.075	0.155	1.19	0.74	2.06
Total N of free amino acids[d]	1,449.7	1,120.7	979.7	457.6	424.0	0.87	0.68	0.94

[a] H, S, D = Healthy (H), sliced (S), and diseased (D) tissues. Sunn., O.L. = Sunnyside and Orange Little
[b] Ornithine may also be contained.
[c] There is a possibility that this is ethanolamine.
[d] γ of N per g fresh weight.

at the last dialysis because it might accelerate aggregation during storage of the extracts in the frozen condition. C: When plant tissues are homogenized, browning often occurs as a result of an oxidation of polyphenols by polyphenol oxidase and subsequent reaction of the quinones with amino acids or proteins. Some workers have prevented browning by using reducing agents as ascorbic acid (2, 19) and cysteine (10). In the healthy tissue adjacent to the infected, the polyphenol oxidase activity and the polyphenols increased and a brown reaction occurred rapidly when the tissue was homogenized (2). From preliminary experiments, 1 % ascorbic acid was found to prevent coloration through the whole extraction procedure. D: Immediately after homogenization the homogenate was dialyzed against the buffer containing ascorbic acid before centrifugation. That pre-dialysis was carried out for two reasons: A: During homogenization some of the ascorbic acid and polyphenols might be oxidized and then could react with the proteins. Such substances should be removed by the pre-dialysis; B: when the centrifuging was done without pre-dialysis some browning occurred which was prevented by pre-dialysis.

Some of the antigenic components shown by the immunochemical tests, such as components A, B, C_s, C_o, and D (fig 1) are considered to be proteins, because they were non-dialyzable, precipitated with ammonium sulfate, and moved on starch gel by electrophoresis and on cellulose ion-exchange columns at different rates. Component D showed peroxidase action.

In response to an infection the respiratory rate is increased in many host plants. We assume that the augmented respiration is coupled to adenosine triphosphate utilizing systems such as protein synthesis; many results support this assumption (31, 34, 38, 43). The data presented in this paper which showed remarkable changes in protein metabolism when sweet potatoes are infected, provide additional evidence for the above assumption. When infected by the pathogen, the host produces polyphenols and coumarins in the healthy tissue adjacent to the infected, and terpenoids in the infected tissue (38, 39). That may be caused through rearrangement of some metabolic pathways which is related to a change in protein metabolism.

The immunochemical tests and other experiments revealed that some protein components were produced in diseased tissue in response to the infection. One component, D, showed peroxidase activity. Following the infection several new peroxidases appear of which D showed the strongest activity on starch gel. It is interesting that an enzyme in diseased tissue was effective as an antigen although it was not detected in healthy tissue. This enzyme could function as a cytochrome related to the respiratory system of diseased tissue even though it was detected as a peroxidase.

Component B probably is present in a larger amount in diseased tissue than is component D. A minute amount of antigen B was found in healthy and sliced tissues. The data indicate that antigen B was produced in a much larger amount in Sunnyside, a resistant variety, than in Orange Little, a susceptible one following infection by the fungus. That the B antigen is closely related to resistance is supported by the recent preliminary studies of the Japanese varieties which show that, like the American varieties, the highly resistant strains produce much more of this component than do susceptible varieties following black rot infection. The component was concentrated by modified cellulose chromatography. The component may be an enzyme related to a metabolic system accelerated by the infection, or it might be a protein having another function than that of enzyme action.

Component A was composed of many subcomponents (A_1, A_2, A′, & A″). The main components, A_1 and A_2, were present in every tissue, and as indicated by the position of their precipitation lines, in a higher concentration than most other antigenic components. Some decrease was found in diseased tissue extracts. It is interesting that some subcomponents as A″, which cross reacted with the main components, were present only in diseased tissue. To clarify the nature and function of these antigens may require a better understanding of the proteins in cytoplasm of both healthy and diseased plant cells. Studies like this are being done with tissue proteins (8, 30).

The new components in diseased tissue, showing distinct immunochemical patterns, may be produced in specific response to infection by the pathogen or may be the result of a response to certain types of general stimulation (39). Tomiyama et al. (34) showed changes in the metabolism of white potato (in response to cutting) which were similar to the reaction following infection by *Phytophthora infestans*. Uritani et al. also showed the similar effect of mercuric chloride treatment to that of the infection on sweet potato metabolism (41). It is possible that component D was produced by slicing, since the component is a peroxidase, and since this enzyme activity was increased by slicing, though less than that in diseased tissue (39, 40). Recent preliminary work with Japanese varieties showed that components B and D were produced in response to both infection and slicing, though their amounts were much larger in diseased tissue.

The synthesis of such proteins in diseased tissue is assumed to have been accomplished by microsomes, the active site of protein synthesis. It is interesting that microsomes, especially the 73 S unit, were increased in diseased tissues as compared to healthy tissue, though it should be verified by additional experiments, that such changes occur really in the living system and are not caused during the extraction.

Simultaneously with changes in the protein pattern of diseased tissue, the metabolism of free amino acids was changed. This may have been influenced also by the acceleration of carbohydrate metabolism characterized as a respiratory increase. The decrease

in asparagine, a reservoir of soluble nitrogen, may be significant in relation to synthesis of proteins.

All data obtained by the immunochemical analysis, by electrophoresis in starch gel, and by modified cellulose chromatography indicate that the fungus induces changes in the proteins of host cells adjacent to the infection site. Some proteins increase in concentration following the infections; new proteins may appear that could not be detected before the infection. One protein that shows a marked change has been characterized as a peroxidase. Thus, in sweet potatoes, resistance to Ceratocystis seems to be correlated with the extent to which protein metabolism is altered by the fungus; the more resistant varieties show a greater change in their proteins than do susceptible varieties following inoculation.

We suggest that resistance in such plants is an active process that involves a change in the protein metabolism of the host. Resistance may involve a special type of protein synthesis which is triggered by the presence of the pathogen. This change in protein metabolism or protein synthesis in the host may be set in motion by substances produced by the fungus or by material from the cells that it first invades. The speed and extent by which the protein metabolism of the host cells surrounding the initial infection site is so altered may determine whether the host is resistant or susceptible.

Summary

We studied metabolic changes in proteins and amino acids of sweet potato root tissue in response to infection by *Ceratocystis fimbriata* by the methods of immunochemistry, electrophoresis in starch-gel, and chromatography on modified cellulose columns. Some soluble proteins increased in tissue adjacent to the infection site; others decreased. Of special interest was the finding of new antigens in cells adjacent to the area invaded by the fungus which could not be detected before infection or were markedly increased in concentration as a result of the adjacent infection. Those new antigens or proteins produced in response to infection were more concentrated in tissue of the resistant Sunnyside variety than in the susceptible Orange Little variety. Resistance in both American and Japanese varieties of sweet potatoes seems to be correlated with the ability of the variety to form such proteins following the initial infection. One such antigenic component that is produced near the diseased tissue was identified as a peroxidase. Another component was concentrated by the methods of protein purification from 35- to 87-fold; its nature and function are unclear. Microsomes, especially the 73 S unit, were increased following infection. Resistance in such plants may be correlated with the ability of the host tissues to alter their protein metabolism in response to the infecting agent. and to then form proteins which cannot be detected or can be found at low levels before the infection.

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STUDIES ON 3-INDOLEACETIC ACID METABOLISM. VII. METABOLISM OF RADIOACTIVE 3-INDOLEACETIC ACID BY PEA ROOTS[1, 2]

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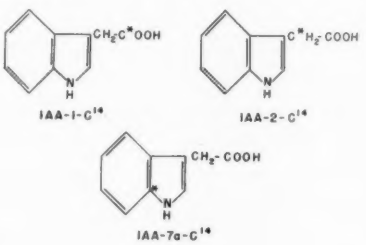
Indoleacetic acid (IAA) applied to pea tissues undergoes both degradation to Salkowski-unreactive substances and conjugation with aspartic acid or ammonia (1, 6). These conjugates have been observed to form only in intact tissues, while in homogenized tissue extracts the IAA molecule is degraded by oxidative decarboxylation, ring opening, or both (8). In previous studies by others on IAA metabolism of intact tissues using radioactive IAA labeled in the side-chain (5, 11) it was found that the carboxyl group (–1–C^{14}) and, after a lag period, the methylene group (–2–C^{14}) yielded respiratory $C^{14}O_2$; the chemical nature of the decarboxylated metabolites remained unknown.

In the experiments reported below, we studied the metabolism of IAA by pea roots using IAA labeled in the side chain (IAA–1–C^{14} or IAA–2–C^{14}) or in the indole nucleus (IAA–7a–C^{14}). We found that indoleacetylaspartic acid was the only metabolite which accumulated in the tissues and that the products of IAA degradation occurred entirely in the culture solution. Three decarboxylation products were recognized, none of which gave a positive indole reaction with the Salkowski or Ehrlich reagent.

MATERIALS & METHODS

PLANT MATERIALS: We used terminal 5 mm root tips of 2-day old Alaska pea seedlings as in previous studies (4).

RADIOACTIVE IAA: We purchased IAA–2–C^{14} (0.20 μc per μmole) from the Atomic Energy of Canada Ltd. and prepared IAA–1–C^{14} (0.09 μc per μmole) and IAA–7a–C^{14} (0.35 μc per μmole) by the method of Robinson (9).



CULTURE CONDITIONS: In all experiments we incubated 12 root tips (about 65 mg total weight) in 20 ml of solution containing IAA (2 μmoles), calcium nitrate (20 μmoles), potassium phosphate pH 5.3 (100 μmoles) and sucrose (280 μmoles) in 125 ml Erlenmeyer flasks. The solutions had been autoclaved for 20 minutes at 20 lb and streptomycin (30 ppm), penicillin (15 ppm) added after cooling. After addition of the tissues, we closed the flasks with sterile stoppers fitted with two glass tubes. One of these tubes extended into the solution and both were closed off. During incubation the flasks were gently shaken (50 strokes/min) in the dark at 22 C for 24 hours.

COLLECTION OF RESPIRED $C^{14}O_2$: At the end of the incubation period the glass tube extending into the solution was opened, the second tube was connected to a sintered-glass aerator dipped into 20 ml of 0.5 N NaOH solution and a gentle stream of air was pulled through the culture flask and the alkali solution for 15 minutes. Aliquots of the NaOH solution, containing the trapped CO_2, were analyzed for C^{14} activity as described below.

TISSUE ANALYSIS: The tissues were homogenized in ethanol and the suspension was made up to a total volume of 10 ml. Aliquots of the suspension were analyzed for total C^{14} activity and for Salkowski-reactive materials as described below. For paper chromatography, half the ethanol suspension was centrifuged and the solids were washed once with ethanol. The supernatant and washings were combined, made alkaline by adding a slight excess of concentrated ammonium hydroxide, and evaporated to dryness on a steam bath. The residue was taken up in a small volume of ethanol and chromatographed on Whatman No. 1 filter paper as described below.

ANALYSIS OF CULTURE SOLUTION: Aliquots of the culture solution were analyzed for total C^{14} activity and Salkowski-reactive materials as described below. For paper chromatography half the solution was saturated with ammonium sulfate, acidified to pH 2.5, and extracted three times with *n*-butyl alcohol. The butyl alcohol extract was made alkaline by adding a slight excess of concentrated ammonium hydroxide and evaporated to dryness on a steam bath. The residue was taken up in ethanol and applied to paper.

SALKOWSKI TESTS: Aliquots of the ethanol tissue suspension were taken to dryness on a steam bath and

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[2] Contribution No. 195.

the residue taken up in 1 ml of 0.1 N sodium bicarbonate solution. Acid ferric chloride, 4 ml, (Salkowski reagent) was added and the Salkowski-reactive materials determined as described previously (2). For analysis of culture solution 4 ml of Salkowski reagent were added directly to 1 ml of culture solution.

C^{14} ANALYSIS: Aliquots of the culture solution and the ethanol tissue suspension were reduced in volume almost to dryness and subjected to chromic acid digestion. The carbon dioxide was collected in an ion-chamber and the C^{14} activity was measured using a vibrating-reed electrometer (Nuclear Chicago "Dynacon", Model 6000). The alkali-trapped $C^{14}O_2$ was liberated by adding acid, collected, and measured in the same way.

PAPER CHROMATOGRAPHY: The papers to which the various extracts had been applied were chromatographed in an ascending solution of isopropyl alcohol: concentrated ammonium hydroxide: water (80:10:15). The chromatograms were dried and exposed to Kodak tri-X film for 4 days. Following radioautography the chromatograms were sprayed either with *p*-dimethylaminobenzaldehyde solution (Ehrlich reagent) or with acid ferric chloride solution (Salkowski reagent) as previously described (6).

RESULTS

Experiments using each of the three preparations of labeled IAA were repeated five times (fig 1 & table I) and the data are presented once more as a metabolic balance sheet in table II.

Figure 1 is a composite radioautograph of seven individual chromatograms. On the far left a single reference standard, containing 0.80 μc of IAA–7a–C^{14}, was applied. The chromatogram shows only IAA. (When ten times amounts were applied, traces of a

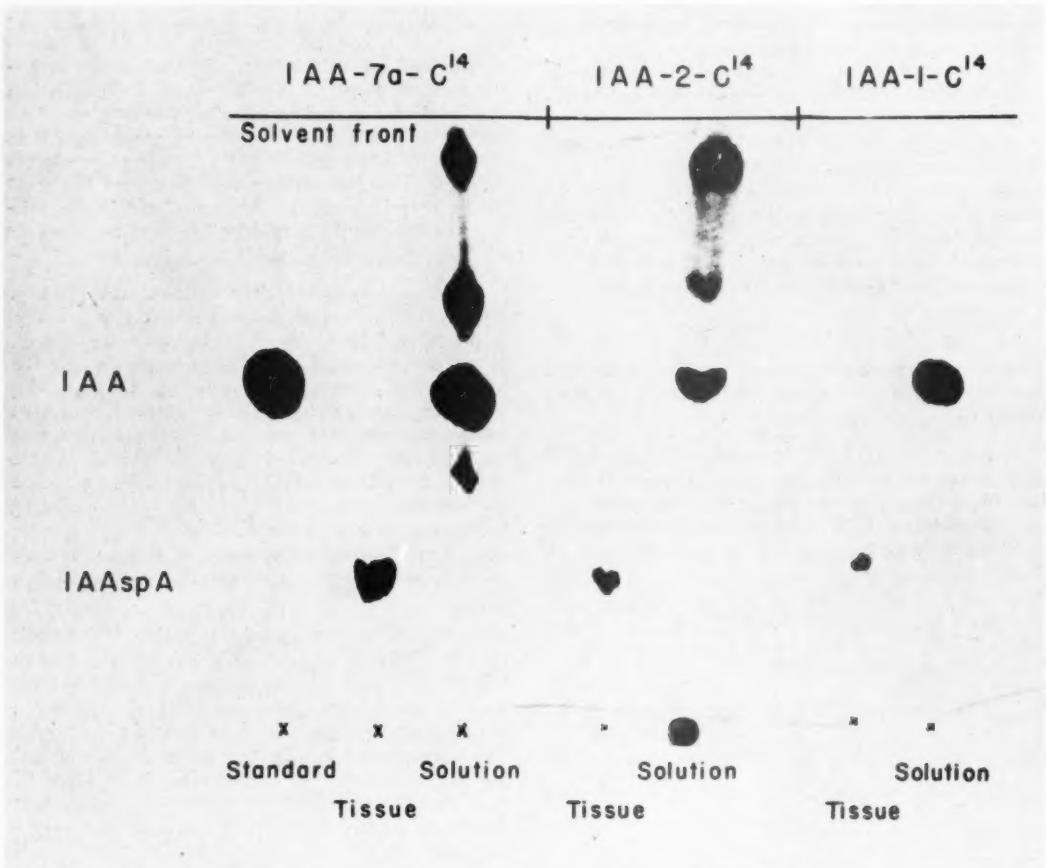

FIG. 1. Radioautograph of indoleacetic acid metabolites: chromatographed on Whatman No. 1 filter paper and exposed to Kodak tri-X film for 4 days; samples applied (*left*) reference standard 0.70 μc of IAA–7a–C^{14}; ethanol extracts of tissues (six root tips, about 33 mg) and *n*-butyl alcohol extracts of culture solutions (10 ml of solution volume).

more mobile component appeared. Judging by the Rf value this was probably skatol, a compound known to result from the spontaneous decarboxylation of IAA). The remaining radioautographs in figure 1 are of tissue and culture solution extracts. The radioautographs of the extracts from tissues exposed to the three preparations of IAA are similar in that they show only a single spot, chromatographically identical with indoleacetylaspartic acid. The decrease in size and intensity of the indoleacetylaspartic acid spot from IAA–7a–C^{14} to the least intense spot with IAA–1–C^{14} corresponds to the decrease in specific activity of these preparations; when the chromatograms were sprayed with Ehrlich or Salkowski reagent the colors produced by these areas were equally intense.

All radioautographs of the extracts of the culture solutions containing the different IAA preparations

TABLE I

QUANTITATIVE ANALYSIS OF INDOLEACETIC ACID METABOLITES

ANALYSIS AFTER INCUBATION	SAMPLE OF IAA–C^{14} APPLIED	% RECOVERY			
		BY C^{14} ANALYSIS		BY SALKOWSKI TEST	
Tissues	–1–	16.3 ± 0.8		18.9 ± 1.6	
	–2–	18.7 ± 1.9	(17.2)	16.3 ± 3.1	(17.1)
	–7a–	16.7 ± 1.6		16.1 ± 1.0	
CO_2 collected	–1–	19.1 ± 3.5		—	
	–2–	0.7		—	
	–7a–	0.2		—	
Culture solution	–1–	59.0 ± 4.2		54.0 ± 3.2	
	–2–	78.0 ± 2.7		52.0 ± 5.1	(52.8)
	–7a–	77.5 ± 3.4		52.5 ± 1.9	
Total recovery of amount applied	–1–	94.4		72.9	
	–2–	97.4	(95.4)	68.3	(69.9)
	–7a–	94.4		68.6	

The values listed are the means and standard errors of five experiments.

TABLE II

DISTRIBUTION OF INDOLEACETIC ACID METABOLITES

ANALYSIS AFTER INCUBATION			QUANTITATIVE ANALYSES RECOVERY OF IAA–C^{14} (% OF AMOUNT ADDED)	DISTRIBUTION OF IAA–C^{14} METABOLITES (% OF IAA–C^{14} METABOLITES RECOVERED)	CHROMATOGRAPHIC ANALYSES (Rf)
Unchanged IAA recovered from culture solution: (Salkowski test)			52.8		0.56
IAA metabolites in tissue:					
IAA (chromatography)			None	None	None
IAAspA (Salkowski test)		17.1			
IAAspA (C^{14} analysis)		17.2	17.2	41	0.23
IAA metabolites in culture solution:					
IAAspA (chromatography)			None	None	None
Decarboxylated IAA					
$C^{14}O_2$ collected (C^{14} analysis of IAA–1–C^{14})			19.1	45	0.94
Metabolites of IAA–2–C^{14} & IAA–7a–C^{14} (Chromatography)					0.70 0.42
Other metabolites: (From difference of unchanged IAA recovered from culture solution by Salkowski test (52.8) & C^{14} analysis corrected for decarboxylated IAA)					
IAA–1–C^{14}	(59.0)	6.2			
IAA–2–C^{14}	(78.0–19.1)	6.1	6.0	14	(Streaks?)
IAA–7a–C^{14}	(77.5–19.1)	5.6			
			95.1		

(For explanations see text)

show unmetabolized IAA. The IAA–2–C^{14} solution contains two other radioactive substances and the IAA–7a–C^{14} solution contains three; as none of these other substances occur with IAA–1–C^{14}, they must be decarboxylated residues. All three chromatograms were somewhat streaked; the streaks are too faint to be seen photographically (fig 1) in the case of IAA–1–C^{14}. These streaks suggest the presence of small amounts of other badly resolved metabolic products (see table II). None of the radioactive areas, except that of IAA itself, gave an Ehrlich test. Even when ten times larger amounts of tissue were treated with IAA there was no indication that these radioactive areas corresponded to indole compounds. Since a purple or blue color with the Ehrlich reagent is considered specific for the presence of indole compounds it seems probable that the radioactive IAA degradation products not only are decarboxylated but also have undergone disruption of the indole nucleus. It is unlikely that these radioactive areas are due to plant products originating from the re-utilization of metabolized C^{14} since none of these compounds occurred on the chromatograms of tissue extracts. The spot at the origin of the IAA–2–C^{14} solution is an artifact.

The quantitative data are shown in tables I and II. The average recovery of C^{14} activity with all samples was 95.4 %. Of this, 17.2 % accumulated in the tissues; from chromatographic evidence this appears to be entirely due to indoleacetylaspartic acid. This value agrees well with the amount of Salkowski-reactive material (17.1 %) found and its additional evidence for the accumulation of only one metabolite in tissue. With IAA–2–C^{14} and IAA–7a–C^{14}, 78.0 % and 77.5 % of the C^{14} activity were found in the culture solution, 52.8 % as Salkowski-reactive material (unmetabolized IAA) and 25.0 % as IAA metabolites which have lost their Salkowski reactivity either by breaking of the indole ring or by decarboxylation of the IAA sidechain or both. Experimental evidence for decarboxylation is apparent from the data with IAA–1–C^{14} where only 59.0 % of the radioactivity could be recovered from the solution while 19.1 % was collected as $C^{14}O_2$. $C^{14}O_2$ collected from the IAA–2–C^{14} and IAA–7a–C^{14} experiments amounted to only 0.7 % and 0.2 %, respectively. These results provide no direct evidence as to whether decarboxylation precedes ring opening or not. However, since the IAA determined in the culture solution by the Salkowski test accounts for only 52.8 % of the original amount while the radioactivity recovered from the culture solution containing IAA–1–C^{14} accounts for 59.0 % and since, using Student's "t" test, this difference was statistically significant at the 5 % level, we suggest that there is in the solution a small amount of at least one IAA–1–C^{14} metabolite which has not been decarboxylated but has lost its Salkowski reactivity, possibly by ring opening between carbons –2– and –3– of the indole ring. The faint radioactive but not Ehrlich-reactive streak found on the chromatograms of solution extracts with IAA–1–C^{14} could be due to such a poorly resolved metabolite.

Discussion

Over a 24 hour period, 41 % of the IAA metabolized is conjugated with aspartic acid to give indoleacetylaspartic acid which accumulates entirely within the tissues, while the remainder is degraded to apparently non-indolic materials which accumulate entirely outside the tissues. These proportions are in general agreement with those found in our earlier studies (2, 4). The fact that our results differ so markedly from those obtained by Fang et al. (5) has already been ascribed to the presence in our media of calcium ions which are essential to the normal metabolism of IAA by isolated pea roots (4). The importance of maintaining sterile conditions, especially when using media containing sucrose, must be stressed. We have observed on several occasions that media accidentally contaminated by microorganisms may release up to theoretical quantities of $C^{14}O_2$ in 10 to 15 hours from labeled IAA, even when the label is in the benzene ring. Note also that the ratio of degradation to conjugation is not constant, but depends upon the duration of the incubation period and the concentration of applied IAA; with low concentrations of IAA in short-term experiments, IAA chiefly undergoes conjugation (4).

The fact that the degradation products accumulate only in the culture solution does not necessarily imply that degradation takes place extracellularly; it is possible that the products form in the tissues and subsequently diffuse out into the solution. It is most probable that degradation occurs primarily within the epidermis and at the cut surface while conjugation takes place in the remainder of the tissue (4).

While a negative reaction with the Ehrlich reagent cannot be considered as infallible evidence of ring opening, nevertheless these studies suggest that the three products of decarboxylation no longer possess an intact indole nucleus. The estimated presence of small amounts (6 %, see table II) of other Salkowski-unreactive but not decarboxylated metabolites provide some support to the suggestion of Manning and Galston (7) and Stutz (10) that ring opening may precede decarboxylation. The two major, decarboxylated metabolites retained both the –2–C^{14} and the –7a–C^{14} carbon atoms; the third decarboxylated metabolite retained only the –7a–C^{14} carbon atom and gave the smallest spot on the chromatogram; from the data on $C^{14}O_2$ collected, the latter accounted for only about 2.5 % of the decarboxylated products found. The observed metabolic stability of the –2–C^{14} carbon atom may depend on our experimental conditions where the molecules can diffuse back into the culture solution before their complete degradation occurs. These results are in essential agreement with experiments on plum stem cuttings which received a single treatment of IAA–2–C^{14} for only 5 minutes (11); the –2–C^{14} carbon atom began to appear to any extent in the respiratory CO_2 only after a lag period of 24 hours.

Any decision as to whether or not the products of IAA degradation by intact tissues are the same as by

homogenized tissues must await irrefutable chemical identification of the metabolites. With homogenized tissue preparations Manning and Galston reported (7) two products of IAA degradation which gave an orange-yellow color on chromatograms sprayed with the Ehrlich reagent; no such color was found in our study. Stutz (10) with more highly purified tissue preparation and in the presence of the cytochrome system obtained indolecarboxyl aldehyde and two other metabolites which were similar to ours in retaining the –2–C^{14} carbon atom.

Summary

The apical 5 mm root tips of 2-day old Alaska pea seedlings were incubated in solutions containing sucrose, calcium ions, potassium phosphate pH 5.3, and samples of radioactive IAA labeled with C^{14} in one of the following positions: acetic–1, acetic–2, or indole–7a. Analysis after 24 hours revealed that the tissues contained indoleacetylaspartic acid as the only detectable metabolite while the culture solution contained no indoleacetylaspartic acid. Instead the solution contained unmetabolized IAA and three Ehrlich-negative decarboxylation products: two major ones labeled in position –2– and –7a– and one minor metabolite labeled only in position –7a–. At a concentration of 2 μmoles of IAA in 20 ml, metabolism proceeded as follows in 24 hours: 52 % of the applied IAA remained unchanged in the culture solution; 17 % was conjugated with aspartic acid; 19 % was decarboxylated, and about 6 % was degraded without decarboxylation.

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INHIBITORS OF THE HILL REACTION [1, 2]

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Many commercially important herbicides seem to kill plants by inhibiting photosynthesis. Wessels and van der Veen have shown that leaves or parts of leaves treated with phenylureas irreversibly lose all ability to assimilate CO_2. They also found that chloroplasts treated with phenylureas are unable to reduce oxidants such as 2,6-dichlorophenolindophenol in the light (20). A wide variety of phenylcarbamates similarly inhibit photochemical reductions by chloroplast (the Hill reaction) although somewhat higher concentrations of these carbamates are required (20, 11). Recently a number of acylanilides have been introduced as herbicides. These, too, interfere with the Hill reaction (12). The phenylcarbamates, phenylureas, and acylanilides are, of course, closely related chemically: all are anilides of carboxyacids. However, the very dissimilar herbicide Simazine [2-chloro-4,6-bis(ethylamino)-s-triazine] also inhibits the Hill reaction and does so at low concentrations (13).

It has been possible to identify more specifically the process which is inhibited in chloroplasts by phenylureas. Bishop discovered that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibited photosynthesis but not photoreduction in the alga Scenedesmus (3). Since photoreduction differs from photosynthesis in that hydrogen gas replaces water as the ultimate electron donor, it seems reasonable to suppose that DCMU interferes with the mechanism of water oxidation—that is, with the process leading to the production of molecular oxygen. This hypothesis has been supported by experiments with chloroplasts from higher plants. Thus, Jagendorf showed that ATP formation (and therefore probably electron flux) was inhibited in chloroplasts by 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) when flavin mononucleotide (FMN) was the electron carrier but not when N-methylphenazonium ion (PMS) was the carrier (8). Later Krall, Good, and Mayne demonstrated that the FMN catalysed process involved the production and reutilization of molecular oxygen whereas the PMS catalysed process did not (10). Apparently reduced PMS can replace water as electron donor in a reaction analogous to photoreduction. In accordance with Bishop's hypothesis, the process which is independent of the production of molecular oxygen is also resistant to CMU inhibition.

In the work described below the chloroplast-inhibiting herbicides and a large number of related substances were investigated. This report deals with four aspects of the problem:

I. More than 200 acylanilides, thioacylanilides, acylamides, ureas, and thioureas were prepared and identified. About half of these were not described in the readily accessible literature and probably many are new.

II. The work of Wessels and van der Veen (20) and of Moreland et al. (11, 12) on the relationship of inhibitor structure to inhibitor potency has been extended. Inhibition was routinely measured as a decrease in the ability of isolated chloroplasts to reduce ferricyanide in the light.

III. The Jagendorf criterion was used to determine the site of inhibition. Inhibition of the photochemical formation of ATP with FMN as catalyst was compared with the inhibition of ATP formation with PMS as catalyst. This test was applied to representative acylanilides, alkylureas, and triazines as well as to the previously examined phenylureas.

IV. An attempt was made to correlate the bonding potentials of the imino hydrogens of the various inhibitors with inhibitor structure and biological activity. Wessels and van der Veen (20) have suggested that the carbonyl oxygen of the cyclopentanone ring of the chlorophyll molecule may form hydrogen bonds with the imino hydrogen of phenylcarbamates and phenylureas, thus disrupting the photochemical apparatus of the chloroplast. For reasons to be discussed, we do not consider that this particular bond could account for the observed inhibitions. Nevertheless some of the most striking properties of anilides and other amides (high melting points, low vapor pressures, preference for polar solvents, etc.) are directly related to intermolecular hydrogen bonding. Therefore it seems quite probable that these inhibitors become attached by hydrogen bonds to the as yet unidentified active site. Since the formation of a hydrogen bond results in a replacement of one of the characteristic N-H infra-red absorption bands by an N-H ... O band at a longer wave length, the bonding of the imino hydrogen of representative anilides and triazines to various carbonyl groups was observed by infra-red spectroscopy.

[1] Received April 10, 1961.

[2] Contribution No. 194, Pesticide Research Institute, Research Branch, Canada Agriculture, University Sub Post Office, London, Ontario.

Materials & Methods

I. Syntheses of Inhibitors. Acylanilides were prepared by treating the appropriate free aniline either with acid anhydrides (acetic, propionic, butyric, & isobutyric) or with acid chlorides. The anhydrides or chlorides of the acids were added slowly to a benzene solution of the aniline, and the resulting suspension was boiled for a few minutes. Those anilides which were insoluble in benzene were filtered off, dissolved in alcohol, treated with Norite, and recrystallized from alcohol or, more often, from alcohol and water. Anilides which were soluble in benzene were precipitated by adding *n*-hexane. To bring out the anilides of the higher fatty acids large amounts of hexane and cooling to about −15 C were necessary. The acid chlorides were prepared by heating the acids under reflux with an excess of thionyl chloride and were used without purification after the bulk of the unreacted thionyl chloride had been removed by distillation. Isobutyrylamides were prepared in a similar manner except that no heating of the alkylamine-acid anhydride mixture was required. Formanilides were prepared by heating the anilides under reflux in a large excess of formic acid.

4-Chloro- and 3,4-dichloroanilides of thiopropionic acid were prepared by treating the corresponding oxygen anilides with P_2S_5 (9). The solubility of the thioanilides in dilute KOH was used to facilitate their isolation. They were recrystallized from a solution of benzene and *n*-hexane.

Asymmetrical ureas were prepared either by the action of dimethylcarbamyl chloride on the appropriate amine (3,5-dichloroaniline, 3-chloroaniline, octylamine, benzylamine, cyclohexylamine) at room temperature or by the action of alkylamines on the isocyanates derived from 3,4-dichloroaniline and 2-chloroaniline. Note that unless heat is avoided in the reaction involving dimethylcarbamyl chloride, a rearrangement takes place which results in the formation of symmetrical ureas. The reaction of dimethylcarbamyl chloride with anilines is very slow at room temperature, requiring one to several weeks; with 2-chloroaniline it is prohibitively slow and even at room temperature yields predominantly the symmetrical 1,3-bis(2-chlorophenyl)urea.

Asymmetrical thioureas were prepared by the action of alkylamines on 3,4-dichlorophenyl isothiocyanate.

Symmetrical diphenylureas and diphenylthioureas (most of which were too insoluble to test) were prepared by prolonged heating of the appropriate aniline with dimethylcarbamyl chloride (see above) or dimethylthiocarbamyl chloride.

Alkylamines, anilines, and acids used in this investigation were obtained from commercial sources except 3,4-dichlorophenylacetic acid which was prepared by the Arndt-Eistert synthesis (1) from 3,4-dichlorobenzoyl chloride. The phenyl isocyanates and phenyl isothiocyanates were prepared by slowly adding the corresponding aniline to solvents containing an excess of phosgene or thiophosgene (2).

The melting point of each of the potential inhibitors was determined on a melting point block. This block had been calibrated with standard pure substances having well-recognized melting points. Melting points so obtained were identified with those in the literature whenever possible. The only major difference involved 1-benzyl-3,3-dimethylurea (found 76–77°, literature 166°); the substance described in the literature (19) is probably 1,3-dibenzylurea, mp 168°. The melting point of another of our products, N-isobutyryl-2,6-dimethylaniline, could not be established. This substance softened and sublimed over a range of more than 100°. Some of the substituted ureas also had abnormal (double?) melting points. In these instances we have followed the literature in reporting only the higher temperature. (table I).

When no description of the substance could be found (or if there was reason to question the literature) the identity of the substance was confirmed by elemental analysis. (table I).

II. Determination of Relative Potencies of Inhibitors. Chloroplasts from young pea plants (*Pisum sativum* L.) were isolated in the following manner: Freshly picked leaves were ground in a chilled mortar with an ice-cold buffer consisting of 135 g sucrose, 3.0 g tris(hydroxymethyl)aminomethane, and 1.0 g sodium chloride per liter, adjusted to pH 7.95 with 1 N sulfuric acid. The homogenate was filtered through glass wool into ice-encased centrifuge tubes. After centrifugation for 3 minutes at about 4,000 times gravity, the supernatant was discarded and the chloroplasts were resuspended in more cold buffer. The suspension was transferred to other ice-encased centrifuge tubes and was again centrifuged for 2 minutes at 4,000 times gravity. The chloroplasts were then suspended in a minimum volume of buffer and the suspension was filtered through glass wool again to remove clumps. The final dense suspension was stored briefly in a flask which was deeply immersed in chopped ice and water.

The reaction mixture (final volume 2.0 ml) consisted of the above mentioned buffer, methylamine hydrochloride (24 μmoles), potassium ferricyanide (1.0 μmole), and an amount of the chloroplast suspension containing about 30 μg chlorophyll. Ferricyanide reduction was followed spectroscopically in an apparatus described elsewhere (6). Reaction temperatures were held at about 15 C by cooling the cuvette holder with rapidly circulating tap water. An intense beam of light from a 300 w projection lamp was passed through a red glass filter and directed against the frosted side of the reaction vessel, a 1 cm cuvette. The inhibitors to be tested were dissolved in alcohol. Different concentrations were prepared, usually by a process of serial dilution. After it had been ascertained that 5% alcohol alone had very little effect on the rate of ferricyanide reduction, these alcoholic inhibitor solutions were added to the reaction mixture in amounts not exceeding 0.1 ml. With each inhibitor the concentration which halved the reaction rate was determined. For convenience

TABLE I

AMIDES AS INHIBITORS OF HILL REACTION
GENERAL FORMULA: R-NH-CX-R¹

No.	R	X	R¹	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
	Part I—Anilides from unsubstituted aniline							
1	Phenyl	O	Methyl	3.7	113–114	114		
2	"	O	Chloromethyl	5.0	133–134	134		
3	"	O	Dichloromethyl	4.4	118	118		
4	"	O	Trichloromethyl	5.0	94	94		
5	"	O	Ethyl	4.7	105	103		
6	"	O	1-Chloroethyl	4.1	89–90	92		
7	"	O	1,1-Dichloroethyl	4.4	99–100	101		
8	"	O	Propyl	4.0	94–95	95		
9	"	O	2-Propyl	4.2	105–106	105		
10	"	O	Phenyl	4.0	161–162	160		
11	"	O	2-Methyl-1-propenyl	5.2	127–129		N 8.04	N 8.00
12	"	O	Dimethylamino	5.2	129–130	127–129		
13	"	S	Dimethylamino	2.0	132–133	127		
14	"	O	2-Propoxy	4.1	87–88	90		
	Part II—4-Chloroanilides							
15	4-Chlorophenyl	O	H	2.5	101–102	102		
16	"	O	Methyl	4.3	178–179	179		
17	"	O	Chloromethyl	4.7	169–170	169		
18	"	O	Dichloromethyl	4.7	137–138		Cl 44.5	Cl 44.6
19	"	O	Trichloromethyl	4.6	127–128		Cl 52.2	Cl 52.0
20	"	O	Ethyl	4.5	137	141		
21	"	S	Ethyl	3.2	77–78		Cl 18.3 S 16.0	Cl 17.8 S 16.1
22	"	O	1-Chloroethyl	4.7	112		Cl 32.1	Cl 32.5
23	"	O	1,1-Dichloroethyl	5.1	94–95		Cl 42.0	Cl 42.1
24	"	O	Propyl	4.7	102–103	104		
25	"	O	2-Propyl	5.2	148–150	153		
26	"	O	Phenyl	4.5	192	192–193		
27	"	O	2-Methyl-1-propenyl	5.0	121–122		Cl 17.0	Cl 17.0
28	"	O	2-Methyl-2-propyl	4.5	148–149		Cl 16.7	Cl 16.8
29	"	O	Dimethylamino	6.3	169	171		
30	"	S	4-Chloroaniline	4.5s	178–179		Cl 23.8 S 10.8	Cl 23.9 S 10.7
	Part III—3-Chloroanilides							
31	3-Chlorophenyl	O	H	3.2	55–56			
32	"	O	Methyl	4.5	76	79		
33	"	O	Chloromethyl	5.0	99–100		Cl 34.9	Cl 34.8
34	"	O	Trichloromethyl	5.0	101–102		Cl 52.4	Cl 52.0
35	"	O	Ethyl	5.5	87–88	88–89		
36	"	O	Propyl	5.3	45–46		Cl 17.6	Cl 17.9
37	"	O	2-Propyl	4.8	112		Cl 17.8	Cl 17.9
38	"	O	2-Methyl-1-propenyl	5.0	112–113		Cl 16.9	Cl 16.9
39	"	O	3-Chloroaniline	5.0	246	245	Cl 23.2	Cl 23.0
40	"	O	Dimethylamino	6.3	139–141		Cl 17.9	Cl 17.9
41	"	O	2-Propoxy	4.4				
42	"	O	4-Chloro-but-3-yn-1-oxy	5.1	75–76			

* pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

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TABLE Ia

AMIDES AS INHIBITORS OF HILL REACTION

GENERAL FORMULA: R-NH-CX-R^1

No.	R	X	R^1	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
	Part IV—2-Chloroanilides							
43	2-Chlorophenyl	O	Trichloromethyl	3.7	62		Cl 51.8	Cl 52.0
44	"	O	Ethyl	2.6	92–93	91		
45	"	O	1-Chloroethyl	2.3	59		Cl 32.3	Cl 32.5
46	"	O	Propyl	2.0	80		Cl 17.7	Cl 17.9
47	"	O	2-Propyl	2.2	93–94		Cl 17.7	Cl 17.9
48	"	O	2-Methyl-2-propyl	2.5	76		Cl 16.6	Cl 16.7
49	"	O	2-Methyl-1-propenyl	3.4	88–89		Cl 16.9	Cl 16.9
50	"	O	Dimethylamino	3.3	92–93	95		
	Part V—3,5-Dichloroanilides							
51	3,5-Dichlorophenyl	O	H	3.2	127		Cl 37.5	Cl 37.3
52	"	O	Methyl	4.0s	186	186–187		
53	"	O	Trichloromethyl	5.7	121–122		Cl 58.1	Cl 57.8
54	"	O	Ethyl	5.7	118–120		Cl 32.8	Cl 32.7
55	"	O	2-Chloroethyl	4.5	97–98		Cl 41.9	Cl 42.1
56	"	O	Propyl	4.6	85–86		Cl 30.9	Cl 30.6
57	"	O	2-Propyl	4.8	134–135		Cl 30.5	Cl 30.6
58	"	O	2-Methyl-1-propenyl	4.7	106–107		Cl 29.0	Cl 29.0
59	"	O	Dimethylamino	6.0	163–165		Cl 30.2	Cl 30.4
	Part VI—2,4-Dichloroanilides							
60	2,4-Dichlorophenyl	O	Methyl	2.5s	143–144	144		
61	"	O	2-Propyl	2.8s	124–125	124		
	Part VII—3,4-Dichloroanilides							
62	3,4-Dichlorophenyl	O	H	3.5	108–109	110–112		
63	"	O	Methyl	5.5	122–123	121		
64	"	O	Chloromethyl	6.5	106–107		Cl 44.7	Cl 44.7
65	"	O	Trichloromethyl	6.5	124–126		Cl 57.8	Cl 57.7
66	"	O	Bromomethyl	6.6	99–101		N 4.98	N 4.96
67	"	O	Ethyl	6.8	91–92	91–92		
68	"	O	1-Chloroethyl	6.2	127–129	133–134		
69	"	O	1,1-Dichloroethyl	6.0	110–112		Cl 49.3	Cl 49.5
70	"	S	Ethyl	4.3	71–72		Cl 29.5 S 13.4	Cl 30.4 S 13.7
71	"	O	2-Chloroethyl	5.2	112–113		Cl 42.0	Cl 42.1
72	"	O	2-Propenyl	6.7	120–122			
73	"	O	2-Methyl-2-propyl	6.2	145–146		Cl 28.9	Cl 28.8
74	"	O	Propyl	5.7	79–80	77–78		
75	"	O	2-Propyl	6.2	132–134	135		
76	"	O	3-Pentyl	5.5	125	125		
77	"	O	2-Butyl	6.3	112–113		Cl 28.8	Cl 28.9
78	"	O	Butyl	6.5	73.5	71.5–73		
79	"	O	2-Methyl-1-propyl	5.0	85–86	82–87		
80	"	O	2-Pentyl	7.0	105–106	108–109		
81	"	O	Pentyl	6.5	75–76		Cl 27.3	Cl 27.3

*pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

(Continued on next page)

(Continued from Ia)

TABLE Ib

AMIDES AS INHIBITORS OF HILL REACTION

GENERAL FORMULA: R-NH-CX-R^1

No.	R	X	R^1	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
Part VII—3,4-Dichloroanilides (Continued)								
82	"	O	Hexyl	5.2	61.5	60–61.5		
83	"	O	Heptyl	6.0	42		Cl 24.4	Cl 24.6
84	"	O	Octyl	5.4	69–70		Cl 23.4	Cl 23.5
85	"	O	Nonyl	5.4	70–71		Cl 22.5	Cl 22.4
86	"	O	Phenyl	5.2	145–146		Cl 26.7	Cl 26.7
87	"	O	2-Chlorophenyl	4.8	152–153		Cl 34.8	Cl 34.5
88	"	O	4-Chlorophenyl	5.7	172–173		Cl 35.5	Cl 36.0
89	"	O	2,4-Dichlorophenyl	5.5	156–157		Cl 42.1	Cl 42.4
90	"	O	3,4-Dichlorophenyl	6.0	227–228		Cl 42.3	Cl 42.4
91	"	O	Cyclohexyl	6.0	137–138		Cl 26.0	Cl 26.1
92	"	O	Benzyl	6.8	132		Cl 25.3	Cl 25.3
93	"	O	3,4-Dichlorobenzyl	5.6	186–187		Cl 40.3	Cl 40.7
94	"	O	Phenoxymethyl	5.0	141–142		Cl 24.0	Cl 24.0
95	"	O	2,4-Dichlorophenoxymethyl	5.5	160–161		Cl 39.0	Cl 39.0
96	"	O	2-Methyl-1-propenyl	5.9	103		Cl 29.0	Cl 29.0
97	"	O	3-Phenylpropyl	5.1	74–75		Cl 23.1	Cl 23.0
98	"	O	Trans-2-phenylethenyl	5.2	179–181		Cl 24.7	Cl 24.3
99	"	O	2,4,5-Trichlorophenoxymethyl	5.2	141–142		Cl 43.4	Cl 44.5
100	"	O	2-Naphthylmethyl	5.4	157–158		Cl 21.6	Cl 21.6
101	"	O	1-Naphthylmethyl	5.6	170–172		Cl 21.7	Cl 21.6
102	"	O	Methylamino	7.0	156–157	155.5		
103	"	O	Ethylamino	6.2	175–176	179.5		
104	"	O	Propylamino	5.8	128–129		Cl 28.2	Cl 28.7
105	"	O	Butylamino	6.7	121–122		Cl 26.8	Cl 27.1
106	"	O	Hexylamino	5.5	104–105		Cl 24.9	Cl 24.5
107	"	O	Cyclohexylamino	5.5	183–184	188	Cl 24.6	Cl 24.6
108	"	O	Benzylamino	5.5	171–172		Cl 24.0	Cl 24.2
109	"	O	2-Hydroxylethylamino	4.8	137–138		Cl 28.6	Cl 28.5
110	"	O	Dimethylamino	7.5	156–157	158–159		
111	"	O	Diethylamino	6.8	111–112		Cl 26.3	Cl 26.2
112	"	O	Dipropylamino	4.7	96–97		Cl 24.2	Cl 24.5
113	"	O	Di-(2-propyl)amino	5.3	130–131		Cl 24.4	Cl 24.5
114	"	O	Piperidino	6.8	172–173		Cl 26.1	Cl 26.1
115	"	O	Morpholino	6.4	153–154	157.5	Cl 25.5	Cl 25.8
116	"	O	Di-(2-hydroxyethyl)amino	4.5	153–154	157.0	Cl 24.3	Cl 24.2
117	"	S	Dimethylamino	4.5	161–162	166.0		
118	"	S	Methylamino	3.7	148–150	147.5		
119	"	S	Diethylamino	4.3	95–96		Cl 25.6	Cl 25.6
120	"	S	Ethylamino	3.8	114–115		Cl 28.5	Cl 28.5
121	"	O	Amino	4.7	152–154	155.5		
122	"	S	Amino	3.3	209–210	203–204		
Part VIII—Miscellaneous anilides of isobutyric acid								
123	2,3-Dichlorophenyl	O	2-Propyl	3.0s	108–109		Cl 30.4	Cl 30.6
124	2,5-Dichlorophenyl	O	"	3.0s	137–139		Cl 30.4	Cl 30.6
125	2,4,5-Trichlorophenyl	O	"	4.5	145–146		Cl 39.9	Cl 40.0
126	2,4,6-Trichlorophenyl	O	"	5.0s	151–152		Cl 39.4	Cl 40.0
127	4-Bromophenyl	O	"	5.0	151–152	150–151		

*pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

(Continued on next page)

(Continued from Ib)

TABLE Ic

AMIDES AS INHIBITORS OF HILL REACTION

GENERAL FORMULA: R-NH-CX-R¹

No.	R	X	R¹	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
Part VIII—Miscellaneous anilides of isobutyric acid (Continued)								
128	2-Methylphenyl	O	2-Propyl	2.3	115–116	115–116		
129	4-Methylphenyl	O	"	4.5	108–109	108		
130	3-Methylphenyl	O	"	4.8	82–83	85		
131	2-Methoxyphenyl	O	"	2.0	44		N 7.19	N 7.25
132	4-Methoxyphenol	O	"	5.0s	109–111		N 7.26	N 7.26
133	4-Nitrophenyl	O	"	4.0	167–169		N 13.4	N 13.45
134	3-Nitrophenyl	O	"	3.8	93		N 13.5	N 13.45
135	3-Chloro-4-methylphenyl	O	"	5.8	146–147		Cl 16.8	Cl 16.8
136	2-Methyl-3-chlorophenyl	O	"	2.5s	142–143		Cl 16.8	Cl 16.8
137	2-Methyl-4-chlorophenyl	O	"	2.5s	163–164		Cl 16.8	Cl 16.8
138	3-Nitro-4-methylphenyl	O	"	4.8	106–107		N 12.5	N 12.6
139	1-Naphthyl	O	"	2.0s	147–149		N 6.57	N 6.58
140	5,6,7,8-Tetrahydro-2-naphthyl	O	"	5.0	102		N 6.57	N 6.45
141	4-Dimethylaminophenyl	O	"	3.0	157–158		N 13.8	N 13.6
142	2,6-Dimethylphenyl	O	"	2.7	?		N 7.26	N 7.35
Part IX—Alkylamides & alkylureas								
143	Cyclohexyl	O	2-Propyl	3.3	116–117		N 8.32	N 8.30
144	Benzyl	O	"	2.3	91–92		N 8.05	N 7.92
145	Octyl	O	Dimethylamino	5.6	27–28		N 13.7	N 14.0
146	Cyclohexyl	O	"	4.8	156–157		N 16.5	N 16.4
147	Benzyl	O	"	3.6	76–77		N 15.7	N 15.7

*pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

these concentrations have been expressed on a logarithmic scale. Thus the $\log_{10}$ of the reciprocal of the molar concentration giving 50 % inhibition is referred to as the pI_{50}. The inhibitors with the larger pI_{50}'s are the more potent and a difference in pI_{50} of one unit represents a tenfold difference in potency.

Several points on the concentration-inhibition curve were established for each compound in order to avoid spurious conclusions which might arise from an inadvertent saturating of the medium with inhibitor. (With saturated or transiently supersaturated solutions, adding more inhibitor does not necessarily increase the effective concentration and may even decrease it by initiating crystallization.) Solubility problems did occur with a number of substances. In fact, many of the compounds prepared and tested are not included in this report because it was impossible to achieve concentrations high enough to cause 50 % inhibition. Other substances were doubtful, but by utilizing the fleeting phenomenon of supersaturation often it was possible to obtain the desired level of inhibition. However, to indicate our reservations we have added the letter "s" after the pI_{50} value listed in table I whenever the true inhibitor concentration was in doubt. Fortunately most of the highly active inhibitors presented no problem, either because their water solubilities, although very small, were adequate to supply the extremely low inhibitor requirement or because, at the very great dilution involved, crystallization was so slow as to render the supersaturated state quasi-stable.

III. INHIBITION OF ATP FORMATION. Chloroplasts (60–90 μg chlorophyll) were illuminated in a reaction medium consisting of the pH 7.95 buffer, ATP (10^{-3} M), K_2HPO_4 labeled with P^{32} (10^{-2} M), magnesium sulfate (3×10^{-4} M), hexokinase (3.0 mg), glucose (2×10^{-2} M), either FMN or PMS (10^{-4} M), a trace of horse liver catalase to hasten the decomposition of the hydrogen peroxide formed on oxidation of the reduced flavin, and various amounts of inhibitor in 0.2 ml of alcohol. The total volume was 5.0 ml. The reactions were carried out

in test tubes (15 mm I.D.) supported in a wire rack immersed in a large rectangular glass water bath. Under the bath was a mirror. Light from a 500 w incandescent lamp was directed down on the bath and light from two 300 w reflector lamps was directed against each side of the bath. The temperature of the bath was held constant ± 1 C during the 10 minutes of illumination either by adding ice or by circulating tap water. The reaction temperature in different experiments varied from 3 C to 20 C, although most of the experiments were conducted at about ten Centigrade. After illumination, the reaction mixture was poured into 10 % perchloric acid and the unreacted orthophosphate was extracted as phosphomolybdate by the method of Nielsen and Leninger (15). The organic phosphate (glucose-6-phosphate synthesised by hexokinase from glucose & labeled ATP) was measured as the residual radioactivity of the acidified medium.

IV. Hydrogen Bond Formation. Various acylanilides, acylamides, alkylureas, phenylureas, and triazines were dissolved in chloroform (approximately 0.067 or 0.5 M), in chloroform + 5 % acetone (0.067 M), and in carbontetrachloride (0.067 M). There is a characteristic absorption band of the N-H bond at 2.9 μ (wave number = 3,440 cm^{-1}) and a corresponding absorption band of the N-H . . . O bond near 3.0 μ. Consequently the absorption spectrum between 2.6 and 3.2 μ of each substance in each solvent system was determined with a Perkin-Elmer Model 21 infra-red spectrophotometer.

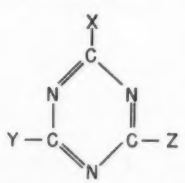
Results

The substances with which the report deals may be classified on the basis of their chemical structures into three unequal groups:

I. Amides having the general formula R-NH-CX-R^1 where X is oxygen of sulfur, R and R^1 are alkyl or aryl, substituted or unsubstituted; R^1 may also be alkylamino, dialkylamino, or arylamino. (See tables I & II).

II. Miscellaneous compounds resembling group I) in that they are also amides but not fitting the general formula, e.g., N-methylanilides, sulfonamides.

III. A small number of symmetrical triazines having the general formula:



where X is chlorine or methoxy, Y and Z are ethylamino, or diethylamino. (See table III).

A. Inhibition of Ferricyanide Reduction. There are several considerations which may throw doubt on comparisons of inhibitor potencies. It is conventional to express the activity of the inhibitor in terms which involve its molar concentration. Now a) if the enzyme-inhibitor reaction is essentially irreversible or b) if the inhibitor's solubilities are such that it partitions between water and the biological material in a manner which strongly favors the latter, then the significant parameter becomes the amount of inhibitor in each chloroplast and not the original concentration of inhibitor in the suspension medium. Unfortunately, the real condition is frequently intermediate and neither the overall inhibitor concentration nor total amount of inhibitor added is uniquely significant. This may make kinetic analysis of the situation almost impossible. We have followed the popular trend and expressed inhibitor potencies as pI_{50}'s, that is, $\log_{10}$ of the reciprocal of the molar concentration causing 50 % inhibition. However, all the inhibitor studies reported in tables I, II, and III were made with the same or nearly the same proportion of chloroplasts to medium. Therefore, the concentration data expressed as pI_{50}'s may be converted into data expressed as amounts of inhibitor per chloroplast or per μg chlorophyll without changing in any way the order of potencies. Of course, this still leaves undecided the question of the proportion of the applied inhibitor actually in the chloroplasts (which proportion may be quite different with different inhibitors) and it must be recognized that these and all similar values for inhibitor potency are the outcome of at least two unrelated properties of the inhibitor: its inherent effectiveness as an enzyme poison and the coefficient of its partitioning between water and chloroplasts.

In order to obtain the maximum Hill reaction rates in our test system, we were very careful to use saturating light intensities and to uncouple the phosphorylating electron transport system with methylamine (6). Under these conditions the uninhibited rates were of the order of 1 millimole ferricyanide reduced per mg chlorophyll per hour.

The reproductibility of the pI_{50}'s in our test varied with different substances. In comparing the potencies of the various substances, it is probably safe to consider a difference of 0.2 as of borderline significance and a difference of 0.3 as quite significant.

I. Acylanilides, Thioacylanilides, Acylamides, Phenylureas, Phenylthioureas, Alkylureas, & Phenylcarbamates Having General Formula R-NH-CX-R^1 (table I).

Part I. Derivatives of unsubstituted aniline. The unsubstituted anilides are not nearly as effective as many of the substituted anilides described below. Among the unsubstituted anilides, the acetyl derivative (No. 1) is a poor inhibitor while the chloroacetyl (No. 2), the trichloracetyl (No. 4) and the β,β-dimethylacrylyl (No. 11) are all fairly good and about equally potent. The well known herbicide, phenyldimethylurea (No. 12), has the same activity as the better acyl derivatives but its sulfur analog (No. 13) is almost inactive. Note that the only carbamate (No. 14) is not very effective as a Hill reaction in-

hibitor although it is a commercial herbicide. The probable mode of action of the phenylcarbamates will be compared with the mode of action of the other anilides in the discussion.

Part II. Derivatives of 4-chloroaniline. These exhibit a wider range of activities than do the unsubstituted anilides; the potencies of the various derivatives do not follow an entirely parallel pattern. The formyl (No. 15) and the thiopropionyl (No. 21) derivatives are particularly poor. The α,α-dichloropropionyl (No. 23), the isobutyryl (No. 25), and the β,β-dimethylacrylyl (No. 27) derivatives are reasonably good. The dimethylcarbamyl derivative (No. 29), (4-chlorophenyldimethylurea, CMU or monuron), is at least 10 times more potent than its nearest competitor.

Part III. Derivatives of 3-chloroaniline. Again the sequence of relative potencies is different in some details. The formyl derivative (No. 31) has very low activity. The acetyl derivative (No. 32) and the isopropylcarbamate (No. 41) are rather poor. The 3-chloro-analog of CMU (No. 40) is, like CMU, outstandingly better than the other members of this group.

Part IV. Derivatives of 2-chloroaniline. Without exception these anilides are feeble inhibitors.

Part V. Derivatives of 3,5-dichloroaniline. Although the average potency is about the same as in the para- and meta-substituted series, these disubstituted anilides do not differ as widely. Again the dimethylurea (No. 59) has the highest activity, but only by a narrow margin.

Part VI. Derivatives of 2,4-dichloroaniline. The two substances in this class resemble 2-chloroanilides. Apparently a chloro group in the ortho position interferes seriously with the mechanism of inhibition.

Part VII. Derivatives of 3,4-dichloroaniline. The 3,4-dichloroanilides are the most active inhibitors we have investigated. Indeed, there is not a single instance of another anilide or alkylamide exceeding the corresponding 3,4-dichloroanilide in potency. Consequently, it seemed appropriate to use 3,4-dichloroaniline as the common moiety in a large number of substances to determine how the structure of the rest of the molecule affected the inhibitory functions. Admittedly, this investigation was not very systematic but, unfortunately, no principles on which to base a systematic study were apparent. Even now, widely applicable generalizations relating activity to the structure of the acyl moiety of the anilides elude us. However, it is possible to make certain generalizations of limited applicability:

Among the anilides of aliphatic acids, inhibitor activity is a periodic function of the chain length of the acid (See fig 1). There is a maximum at the propionyl derivative (No. 67), another maximum

TABLE II

ANILIDES OF ISOBUTYRIC ACID AS HILL REACTION INHIBITORS

No.	Position occupied on benzene ring					pI_{50}*
	2	3	4	5	6	
9						4.2
25			Cl			5.2
37		Cl				4.8
47	Cl					2.2
61	Cl		Cl			2.8
57		Cl		Cl		4.8
75		Cl	Cl			6.2
123	Cl	Cl				3.0
124	Cl			Cl		3.0
125	Cl		Cl	Cl		4.5
126	Cl		Cl		Cl	5.0s
127			Br			5.0
128	CH_3					2.3
130		CH_3				4.8
129			CH_3			4.5
142	CH_3				CH_3	2.7s
132			CH_3O			5.0s
131	CH_3O					2.0
133			NO_2			4.0
134		NO_2				3.8
135		Cl	CH_3			5.8
136	CH_3	Cl				2.0
137	CH_3		Cl			2.5s
138		NO_2	CH_3			4.8
141			$(CH_3)_2N$			3.0

* pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction. "s" following pI_{50} indicates doubts concerning the actual amount of inhibitor in solution.

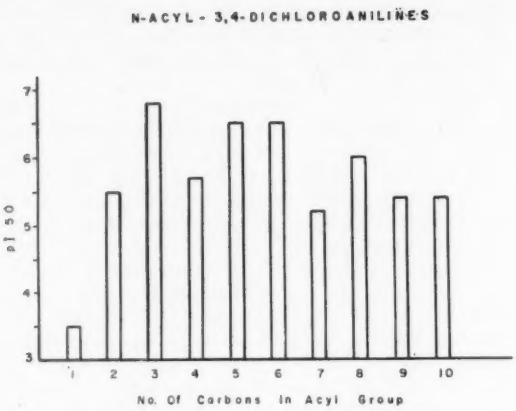


FIG. 1. The effectiveness of 3,4-dichloroanilides as inhibitors of the reduction of ferricyanide by illuminated chloroplasts. pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration giving 50 % inhibition. The abscissa numbers refer to the carbons in the normal acyl moiety.

which includes the valeryl (No. 78) and the caproyl (No. 81) derivatives, and a third lower maximum at the octanoyl derivative (No. 83). Branched or substituted aliphatic acids somewhat resemble the parent straight chain acid. Thus the methylpropionic (isobutyric) anilide (No. 75), like the propionyl derivative, is considerably more active than the butyric anilide (No. 74), while α-methylbutyric acid yields an anilide (No. 77) which is intermediate (perhaps because it is also α-ethylpropionic acid?). In this connection, an interesting substance is the 3,4-dichloroanilide of α-methylvaleric acid, the herbicide Karsil (No. 80). This is one of the most effective inhibitors of the Hill reaction described. It is conceivable that Karsil owes its unusual potency to the fact that α-methylvaleric acid is also α-butylpropionic acid; in a sense Karsil sits on both the first and second maxima shown in figure 1.

Replacing a methyl group in the aliphatic acid by a chlorine atom, despite the inevitable major rearrangement of electron densities, results in surprisingly little change in the activity of the anilide. The chloroacetanilide (No. 64) resembles the highly active propionyl derivative (No. 67), the anilide of β-chloropropionic acid (No. 71) is similar to the anilide of *n*-butyric acid (No. 74), and the α-chloropropionyl derivative (No. 68) has the same activity as the isobutyryl derivative (No. 75). On the average, however, the chloro- acids form 3,4-dichloroanilides of slightly lower activity than do their methyl counterparts. The effect of replacing a methyl group by a bromine atom seems to be similar since the bromacetanilide (No. 66) is also comparable to the propionic anilide in activity.

Polar groups sharply reduce the activity of the inhibitor. Thus, 3-(3,4-dichlorophenyl)-1-(2-hydroxyethyl)- and 3-(3,4-dichlorophenyl)-1, 1-bis(2-hydroxyethyl)- ureas (No. 109 & No. 116) are poor inhibitors. On the other hand, the dehydrated version of the latter compound, in which the two hydroxyl groups are replaced by a relatively non-polar ether bridge (No. 115), is 100 times more active than its polar counterpart. It is uncertain whether this inactivity of polar substances should be explained in terms of enzyme affinities or on the basis of partitioning and penetration; inactivity could result from the fact that such inhibitors fail to enter the lipid-rich chloroplasts in significant amounts.

The thiopropionyl-3,4-dichloraniline (No. 70) and the 3,4-dichlorophenyl-thioureas (No. 117 to No. 120) are from 300 to 2,000 times less effective than the oxygen analogs.

The important herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea or DCMU (No. 110) is the most inhibitory substance we have encountered. In this respect, the 3,4-dichloroanilides resemble the other anilides and alkylamids examined: the dimethylcarbamyl derivative is always the most active of the derivatives tested. However, other 3,4-dichloroanilides are also very good inhibitors; some of their activities approach that of DCMU. Among these are the corresponding monomethylurea (No. 102), the diethylurea (No. 111), the anilide of α-methylvaleric acid (Karsil, No. 80), the anilides of propionic (No. 67) and phenylacetic (No. 92) acids, and the piperidino compound (No. 114).

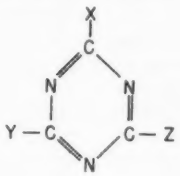
Part VIII. Anilides of isobutyric acid (table II). Isobutyric acid was chosen to contribute the common acyl moiety when a study of the influence of the amine moiety on inhibitor potency was undertaken. The reasons for this choice were the availability of isobutyric anhydride and the simplicity of its use in forming amides, the desirable crystal characteristics of most of the amides, and the moderately high activity of most of the isobutyranilides already tested.

The data, most of which are brought together in table II, may be summarized as follows: When chlorine atoms, bromine atoms, methoxy groups, or methyl groups replace the para- and meta-hydrogens of the aniline, there is a marked increase in activity. The effect is greatest with chlorine, slightly less with bromine and methoxy, and again slightly less with methyl. Meta-para disubstituted compounds are doubly activated while meta-meta compounds are not. On the other hand, any ortho substitution causes a striking reduction in activity. The adverse effect of an ortho-chloro can be overcome in part by adding a meta-para pair as in the 2,4,5-trichloranilide (No. 125). Surprisingly, two ortho-chloro atoms reduce the activity much less than one does. This may be seen by comparing the 2,4-dichloroanilide (No. 61) with the 2,4,6-trichloroanilide (No. 126). Moreland and Hill have observed a similar relationship between derivatives of 2-chloro- and 2,6-dichloroaniline (11). The same is not true of two ortho methyl groups; the 2-methylanilide (No. 128) and the 2,6-dimethylanilide (No. 142) are both exceedingly poor inhibitors. Nitro groups, either meta or para, reduce the activity by a small amount while the dimethylamino group in the para position almost abolishes the inhibitory ef-

TABLE III

TRIAZINES AS HILL REACTION INHIBITORS

GENERAL FORMULA



X	Y	Z	pI_{50}*	TRADE NAME
Cl	Ethylamino	Ethylamino	6.4	Simazine
Cl	Isopropylamino	Isopropylamino	6.3	Propazine
Cl	Ethylamino	Diethylamino	4.7	Trietazine
Cl	Isopropylamino	Diethylamino	4.9	Ipazine
Cl	Isopropylamino	Ethylamino	6.6	Atrazine
CH_3O-	Ethylamino	Ethylamino	5.7	Simetone
CH_3O-	Isopropylamino	Isopropylamino	5.8	Methoxypropazine

* pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration giving 50 % inhibition of ferricyanide reduction.

fect (No. 141). The 3,4-cycloalkyl-substituted anilide, N-isobutyryl-5,6,7,8-tetrahydro-2-naphthylamine (No. 140) is a good inhibitor as might have been predicted, and the 2,3-disubstituted anilide, N-isobutyryl-1-naphthylamine (No. 139) has the low activity which is almost always associated with ortho-substituted substances.

The steric and other effects of ortho substitution will be discussed below under the topic of hydrogen bond formation.

Part IX. Alkylamides & alkylureas. The greater part of this paper deals with acylanilides and phenylureas, partly for historical reasons and partly because they seem to be more active than any alkylamide or alkylurea yet tested. Indeed the amides formed from the smaller alkylamines, such as propylamine, ethylamine, and methylamine, are completely inactive. However, *n*-octylamine (No. 145) and cyclohexylamine (Nos. 143, 146) yield ureas and amides which are fair inhibitors. No derivatives of higher amines have been prepared to date, nor has the possibility of using branched or substituted aliphatic amines been investigated.

II. N-METHYLANILIDES & SULFONAMIDES. N-methyl-N-chloracetylaniline was the only secondary amide tested during this investigation. (Melting points; found 68 C, literature 70 C). It was a very poor inhibitor with a pI_{50} of 2.3. This agrees with the observation of other workers who tested other N-methylaniline derivatives (*20*, 11). Apparently an imino hydrogen is required for inhibition.

Several sulfonamides were prepared and tested. Two of these, the *p*-toluene-sulfonyl derivatives of *n*-octylamine (mp 55 C) and 3,4-dichloroaniline (mp 144 C), were fair inhibitors having pI_{50}'s of 4.5. Since no further tests were made on these compounds, it is not known if sulfonamides and the amides of carboxyacids inhibit the same biological processes.

TABLE IV

INHIBITIONS OF ATP FORMATION IN FMN & PMS CATALYSED SYSTEMS

No.	NAME	pI_{50} WITH FMN (SPECIFIC INHIBITION)	pI_{50} WITH PMS (UNSPECIFIC INHIBITION)
2	N-Chloracetylaniline	4.3	< 2.0
29	3-(4-Chlorophenyl)-1,1-dimethylurea (Monuron, CMU)	6.2	< 4.0
63	N-Acetyl-3,4-dichloroaniline	4.8	3.5
69	N-Propionyl-3,4-dichloroaniline	6.2	4.0
64	N-Chloracetyl-3,4-dichloroaniline	5.8	4.5
65	N-Trichloracetyl-3,4-dichloroaniline	6.3	4.8
80	N-(2-Methylvaleryl)-3,4-dichloroaniline (Karsil)	7.0	4.9
110	3-(3,4-Dichlorophenyl)-1,1-dimethylurea (Diuron, DCMU)	7.2	< 4.0
145	3-Octyl-1,1-dimethylurea	4.6	3.2
	2-Chloro-4,6-bis(ethylamino)-*s*-triazine (Simazine)	5.6	< 3.5
	2-Chloro-4-(2-propylamino)-6-ethylamino-*s*-triazine (Atrazine)	6.2	< 3.5
	2-Chloro-4,5-bis(2-propylamino)-*s*-triazine (Propazine)	5.7	< 3.5

III. Herbicidal Triazines (table III). The most active triazine inhibitors are about as effective as the average 3,4-dichloroanilide and about 10 times less active than the best of the anilides. Not until a more diverse group of triazines has been examined will it be possible to establish reliable rules for predicting the potency of these inhibitors from their molecular structures. On the basis of the limited information provided here, two tentative generalizations are presented: Chlorine atoms are appreciably better than methoxy groups, and two imino hydrogens are very much better than one. It is interesting to speculate as to the significance of the common requirement for imino hydrogens in these apparently very different types of molecules—the triazines and the anilides.

Fig. 2

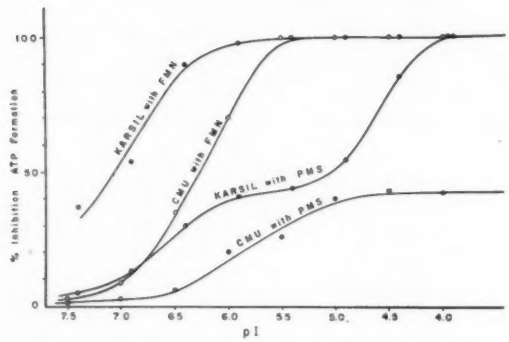


Fig. 3

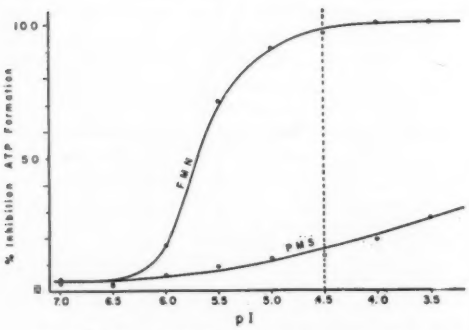


Fig. 2. The inhibition of photophosphorylation by the 3,4-dichloroanilide of α-methylvaleric acid (Karsil) and the 4-chloroanilide of dimethylcarbamic acid (CMU). Electron transport was mediated either by riboflavin-5-phosphate (FMN) or by N-methylphenazonium ion (PMS). pI is the $\log_{10}$ of the reciprocal of the inhibitor concentration.

Fig. 3. The inhibition of photophosphorylation by 2-chloro-4,6-bis(isopropylamino)-*s*-triazine (Propazine). Concentrations to the right of the dotted line represent supersaturated solutions. FMN, PMS, and pI have the same significance as in figure 2.

B. *Localization of Site of Inhibition.* (Figs 2 & 3 & table IV). It has been known for several years that CMU inhibits the formation of ATP by illuminated chloroplasts when either ferricyanide or FMN is the electron acceptor but not when *N*-methylphenazonium ions (PMS) are the acceptors. It was not surprising, therefore, to find that the chemically related acylanilides such as Karsil (No. 80) exhibited a similar behavior. Actually Karsil has two inhibitory functions, one requiring at least 100 times more inhibitor than the other. (See the bimodal Karsil curve in fig 2). Apparently the chloroplast preparation employed for figure 2, unlike most preparations, was unable to by-pass completely the oxygen-producing system in the presence of PMS. Consequently at very low Karsil concentrations all of the FMN-catalysed ATP formation and a part of the PMS-catalysed ATP formation were inhibited. With increasing inhibitor concentration no further increase in the inhibition of the PMS-catalysed system occurred until quite high concentrations had been reached. Probably the curve for CMU inhibition also would have risen again had it been possible to achieve the requisite high concentration. We shall refer to these two types of inhibition as specific (at low concentrations) and unspecific (at high concentrations).

In table IV is shown the specific and unspecific potency of several inhibitors. [The reader should be warned that the data on unspecific inhibition (pI_{50}'s with PMS as catalyst) are quantitatively unreliable because of the limited solubilities of the inhibitors: concentrations quoted are calculated on the basis of the amount of inhibitor added to the medium and ignore the possibility of saturation of the medium. This problem was particularly severe with the very insoluble DCMU & triazines.] If the Jagendorf criterion is a reliable guide to the nature of the process inhibited, we must conclude that each of the twelve inhibitors listed in table IV—and presumably most of the large number of inhibitors with which this paper is concerned—are primarily inhibitors of the mechanism of water oxidation. As already indicated, the chemical similarity of the phenylureas, phenylcarbamates, and acylanilides is such that a common mechanism of inhibition was not unexpected. However, the similar pattern of inhibition with the triazines was a surprise. Errors arising from the solubility problems mentioned above cannot have misled us since Simazine, Propazine (fig 3), and Atrazine inhibited ATP formation in the FMN system completely at concentrations which caused at most a few percent inhibition of the PMS system.

C. *Hydrogen Bond Formation* (Figs 4 & 5). The inhibiting substances described in this paper are very stable compounds. They react only with powerful chemical reagents and many of them resist degradation by the enzymes of plants and bacteria. Pre-

sumably, they are absorbed and held on a catalytic surface such as the active site of an enzyme by physical forces rather than by chemical reactions. [Note that phenylurethane, the classical narcotic or surface-blocking inhibitor of Warburg (18), belongs to the class of anilides with which we have been dealing. Indeed, it was one of the chloroplast inhibitors investigated by Wessels and van der Veen (20)]. A likely binding force, one able to hold substances such as these reasonably firmly, is the electrostatic attraction between the positive imino hydrogen atom of the inhibitor and the negative carbonyl oxygen of a polypeptide chain. With the amide and anilide inhibitors, this force could be augmented by the reciprocal attraction between the inhibitor's carbonyl oxygen and one of the protein's imino hydrogens.

In non-aqueous solutions of anilides, dimers and higher aggregates are produced, the imino hydrogen forming an electrostatic bond with the carbonyl of another like molecule. There is, therefore, an amount of bonding which depends on the concentration of the anilide, on the electron densities throughout the molecule which determine the electro-positivity of the hydrogen and the electro-negativity of the oxygen, and on steric considerations which may limit the accessibility of either the hydrogen or the oxygen. To determine the relative bonding potentials of the imino hydrogens of the various inhibitors without the complication of variations of the carbonyl oxygen, we added an excess of acetone to some of the solutions. With an excess of a common carbonyl compound present, differences in the amount of bonding should de-

Fig. 4

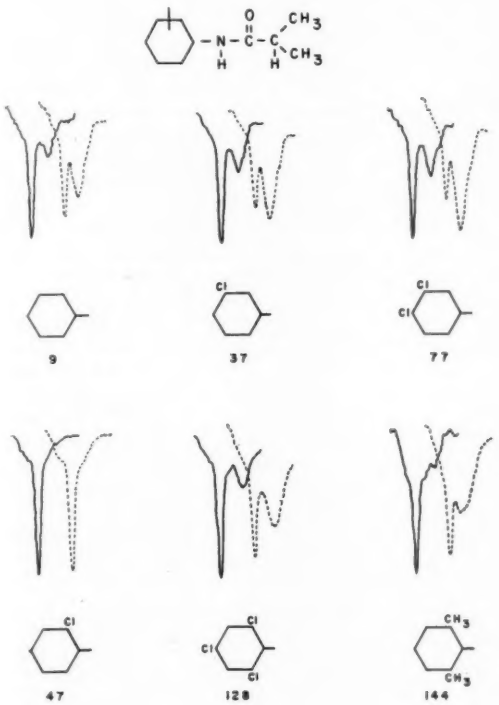
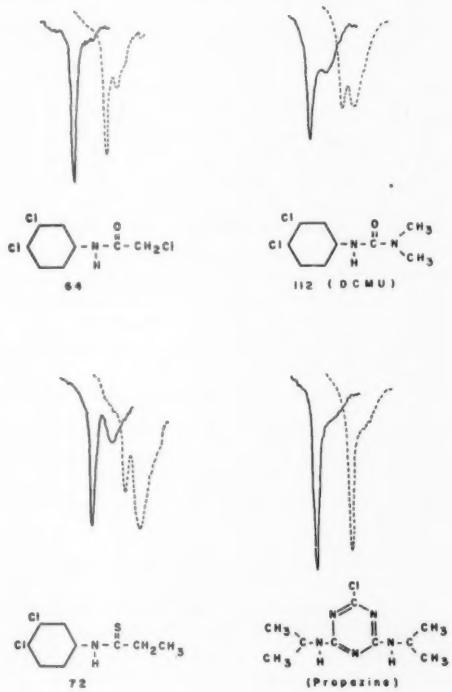


Fig. 5



FIGS. 4 & 5. The absorption spectra of various anilides and Propazine between 2.6 μ and 3.2 μ. The solid lines show the absorption of 0.67 M solutions in chloroform and the dotted lines show the absorption by the same substances when the chloroform solvent contained 5 % acetone. For clarity of illustration the dotted curves have been displaced to the right; actually the narrower peaks on the left of both the solid and dotted curves represent absorption by the N-H bond and are at the same wave length. The broader peaks to the right, which are invariably higher in the dotted acetone curves, represent absorption by the N-H . . . O bond.

pend exclusively on differences in the nature and position of the imino hydrogen. To investigate the characteristics of the imino hydrogens of triazines, which have no carbonyl oxygen of their own, it was obviously also necessary to add an outside source of electronegative groups such as acetone.

Hydrogen bonding was measured as a decrease in the N-H absorption in the region about 3,440 cm^{-1} and the appearance of N-H . . . O absorption about 3,340 cm^{-1}. Twenty-five acylanilides, thioacylanilides, phenylureas, phenylthioureas, and triazines were studied. The findings are summarized in the following generalizations:

I. When the acyl moiety is acetyl, propionyl, or isobutyryl, anilides form hydrogen bonds readily. This tendency is increased in both the 3- and 4-chloroanilides and is even greater in the 3,4- and 3,5-dichloroanilides. The increase in hydrogen bond formation is primarily due to changes in the hydrogen since the same increase occurs in the acetone-containing solvent. Para- and meta-methyl substitution of the aniline has a similar but much smaller effect. All these observations parallel the inhibition data and consequently support the hypothesis that hydrogen bonding plays an important role in the mechanism of inhibition.

II. Also in support of the hypothesis is the fact that the inactive 2-chloroanilides form hydrogen bonds neither between like molecules nor with acetone. This also applies to the 2,4-dichloroanilide (No. 61) and the 2,4,5-trichloroanilide (No. 125). Moreover, the 2,4,6-trichloroanilide (No. 126), which as we have noted, is an unexpectedly effective inhibitor, forms hydrogen bonds to about the same extent as the unsubstituted anilides. The inactive 2-methylanilide (No. 128) and the equally inactive 2,6-dimethylanilide (No. 142) do form hydrogen bonds, but rather reluctantly and the N-H . . . O absorption band is widened and shifted toward the N-H band.

III. Both 4-nitro (No. 133) and 4-dimethylamino (No. 141) anilides form hydrogen bonds but not as freely as the unsubstituted anilides; these substitutions also reduce inhibition, very markedly in the latter case.

IV. On the other hand, halogen substitution of the acyl moiety reduces the tendency to form N-H . . . O bonds without causing a corresponding diminution of inhibition potency. In chloroform solutions the highly active trichloracetyl-3,4-dichloroaniline (No. 65) gives only a very small peak at 3,340 cm^{-1} and the highly active chloracetyl-3,4-dichloroaniline (No. 64) gives none. Even in the acetone solvent the hydrogen bonding tendency is very much reduced.

V. The thioanalogs of acylanilides and of phenylureas have an absorption peak at about 3,340 cm^{-1}. In fact the spectra of the thio compounds and the corresponding oxygen compounds are indistinguishable in the region between 2.6 and 3.2. I was surprised, since sulfur atoms (as in H_2S) do not ordinarily form this type of bond with hydrogens. However, it is well known that the sulfur atoms in compounds such as thioureas and thioamides are exceptionally electronegative. The tendency of the imino hydrogen of thiopropionyl-3,4-dichloroaniline (No. 70) to form bonds with the carbonyl oxygen of acetone is apparently the same as in the oxygen anilides. (See fig 5).

VI. The N-H . . . O peak in the most powerful inhibitors of all, the phenyldimethylureas, is lower than in the acylanilides; both the N-H and N-H . . . O peaks are moved through about 30 wave numbers towards shorter wave lengths.

VII. The imino hydrogens in the triazines do not form bonds with the carbonyl oxygen of acetone to an appreciable extent.

Discussion

Structure & Activity. A variety of substances, including compounds with widely different molecular configurations, are powerful inhibitors of the Hill reaction. Apparently these inhibitors interfere with the same overall process since they prevent the reduction of ferricyanide and FMN by illuminated chloroplasts without seriously hindering PMS-catalysed photophosphorylation. The steric requirements for high activity follow a consistent pattern among the different classes of anilides and alkylamides and, therefore, there can be little doubt that these act at a common site. There is, however, no convincing reason for believing that the triazines must inhibit at the same site. Possibly several catalysed reactions not required in PMS-mediated phosphorylations are involved in the reduction of ferricyanide and FMN. Consequently, it is possible that the poisoning of any one of several catalysts (enzymes?) would explain my experimental results. On the other hand, if the general formula is broadened for the anilide and amide inhibitors, R-NH-CX-R′, to include not only X equals O but also X equals N-, the triazines may be accommodated; moreover the results of experiments employing combinations of phenylureas and triazine are consistent with a single site of inhibition (unpublished). In view of this uncertainty regarding the functional relationship of the triazines to the other inhibitors, the triazines will be disregarded in the following discussion of structure and activity.

Even among the anilides, compounds with quite different structures (e.g. DCMU & Karsil) have similar high activities. However, the apparent low level of specificity on the part of the inhibited catalyst can scarcely be a manifestation of a general low affinity; so few molecules of DCMU are present in an inhibited preparation that a considerable proportion of them must reach, and remain at, the sensitive sites. Although quite different classes of amide inhibitors may share the property of inhibiting at low concentrations, within any one class closely similar compounds may differ sharply. For instance, *N*-valeryl-3,4-dichloroaniline (No. 78) is 30 times more active

than N-isovaleryl-3,4-dichloroaniline (No. 79). Steric considerations seem to be more important in determining the effectiveness of these inhibitors than are factors affecting the distribution of electron densities. Thus chloroacyl-anilines closely resemble the corresponding methyl-substituted acyl compounds. Moreover, para-and meta-substitution of the aniline moiety are equivalent and are opposite in effect to ortho-substitutions; electronically the para and ortho substituted anilines are related and contrast with the meta. This preponderance of steric influences is consistent with a mechanism of inhibition in which the active site is physically obstructed; the important thing is that the inhibitor fit the enzyme well. The great chemical stability of these anilides and the reversibility of the inhibition caused by them (20) argue the same type of inhibition. Electron distributions are more pertinent to chemical reactivity and, therefore, may be expected to play a bigger part in determining the effectiveness of those inhibitors actually reacting with the catalytically active site, e.g., organophosphate inhibitors.

Almost the only feature common to all the Hill reaction inhibitors investigated is the presence of an imino hydrogen. Moreover, there is good reason to believe that this imino hydrogen must not only be present but also accessible if a substance is to have significant inhibitory action. In ortho-chloroanilides, the bulky acyl groups almost certainly lie in the plane of the benzene ring and remote from the also bulky chlorine atom. Such an orientation would force the imino hydrogen into close proximity with the chlorine atom where it would be shielded from interactions with adjacent molecules. Consequently ortho-chloroanilides may be expected to, and do, resemble N-methylanilides in having low melting points, low biological activities, and no hydrogen bonding. In contrast, the imino hydrogen of 2,6-dichloroanilines would be forced into a position away from the plane of the benzene ring and midway between the two chlorines. In this position the imino hydrogen should be, and apparently is, available for hydrogen bond formation and for whatever other functions may be involved in producing the inhibition.

If one were permitted to select his data, it would be possible to obtain a striking correlation between the activity of inhibitors and the tendencies of their imino hydrogen to form bonds with carbonyl oxygen atoms. Substitutions on the benzene ring of the anilides have completely parallel effects on these two properties. For instance, the series 3,4 > 3- and 4->2,4,6-> unsubstituted > 2- applies equally to the hydrogen bonding and the inhibitory action of the chloroanilides. Unfortunately, this beautiful correlation does not apply at all when the acyl moiety is varied. For example, the biologically active chloroacylanilides form hydrogen bonds most reluctantly, and the extremely potent phenyldimethylureas (DCMU, CMU, etc.) form hydrogen bonds much less freely than do the moderately active isobutyrylanilides and the weakly active acetylanilides. Since the author is inclined to follow Wessels in believing that hydrogen bonds should play an important role in anilide inhibitions, he finds these exceptions puzzling. Of course, in this study we may have presented the inhibitor molecules with inappropriate carbonyl oxygens; it may be that hydrogen bonding with an oxygen (or nitrogen?) in a very unusual situation is required if the catalytic site is to be blocked, and, therefore, the *general* bonding potential of the imino hydrogen may be of limited interest. The converse fact that many inactive or almost inactive substances readily form hydrogen bonds is irrelevant since obviously many requirements other than the capacity to form hydrogen bonds must be satisfied if a substance is to be a strong inhibitor.

Site of Action & Photosynthetic Unit. The Bishop-Jagendorf-Krall interpretation of phenylurea inhibition seems plausible in the light of our present knowledge of photosynthesis. Nevertheless, we would do well to remember that our information concerning the mechanisms of photophosphorylation and oxygen production is practically non-existent. Consequently the phosphorylation measurement described above cannot establish either with precision or with certainty the loci of the inhibitions. All that we can say with confidence is this: The anilides (which include phenylureas), the alkylamides, and the triazines have similar modes of action and nothing that we now know is inconsistent with the hypothesis that the mechanism for the oxidation of water to molecular oxygen is the process which is primarily affected.

Wessel's suggestion that the imino hydrogens of phenylureas form hydrogen bonds with the carbonyl oxygen of the cyclopentanone ring of chlorophyll (20), encounters two serious objections. In the first place such a mechanism of inhibition should equally affect photosynthesis, photoreduction, the Hill reaction with ferricyanide or FMN, and the Hill reaction with PMS. As we have seen, this is not the case. In the second place, the better inhibitors are much too effective. The number of moles of DCMU or Karsil required for nearly complete inhibition is smaller than the number of moles of chlorophyll present, by a factor of at least 100. Moreover, this factor of 100 does not allow for the fact that not all of the inhibitor can have reached the site of inhibition. Actually we have shown that at least two-thirds of the DCMU does not even leave the medium under the conditions of our experiments. (This was done both by varying the amount of chloroplast material & by preincubating the medium with chloroplasts which were subsequently removed & discarded). Since the chloroplasts do not remove all of the inhibitor from the medium it follows that the inhibitor-sensitive sites do not remove all of the inhibitor from the other parts of the chloroplasts. In fact, one would expect a higher concentration of inhibitor in the body of the chloroplast than in the medium in view of the solubility characteristics of DCMU and the lipoidal nature of the structural components of the plastids. Consequently, it must be concluded that an unknown but possibly quite small proportion of the inhibitor reaches

its target. This target cannot be chlorophyll unless we postulate one uniquely situated chlorophyll molecule among hundreds or perhaps among thousands. Since excitation energy certainly can be transferred from chlorophyll molecule to chlorophyll molecule many times, there is no reason why there might not be a few special chlorophyll molecules associated with enzymes or substrates to act as a trap for this mobile excitation energy, but it surely stretches credulity also to attribute unique bonding powers to the cyclopentanone carbonyl of these particular molecules.

Nevertheless, the above considerations suggest a possible relationship of the inhibited site to the photosynthetic unit. On the basis of kinetic data [flash saturation of photosynthesis (5), etc.] it has been postulated that a number of chlorophyll molecules collaborate in some way, funnelling their excitation energy or an intermediate chemical product of the excitation energy through a common channel. This photosynthetic unit may have a morphological basis or it may have a purely statistical basis in nature: according to Rabinowitch (16) "the now most plausible interpretation of these phenomena is in terms of an enzymatic component ordinarily present in concentrations 1/300 to 1/2400 that of chlorophyll". The interesting point is that the unit may consist of about 300 molecules of chlorophyll, and the number of units is, therefore, of the same order as the number of molecules of DCMU which we can reasonably expect to be at the inhibited sites when inhibition is practically complete. Unfortunately, the number of photosynthetic units is uncertain and estimates of the number of effective inhibitor molecules are necessarily very crude. Therefore, there is a high probability that the correspondence of the numbers is altogether fortuitous.

Herbicidal Activity & Mechanism of Killing. We are now experimenting to determine the effectiveness of these compounds as inhibitors of the in vivo process of photosynthesis. We are employing excised bean leaves according to the method of Minshall (14). Our preliminary results indicate that the effectiveness of the chemicals in the intact organ is only roughly correlated with in vitro potency. Thus, the thioureas are much too active in vivo and the better acylanilides are not nearly active enough. On the basis of analogy to the well known conversion of P = S to P = O in organophosphate metabolism, we suggest that the thioureas may be fairly rapidly transformed into their oxygen analogs in the plant. The unexpected low toxicity of the acylanilides could be explained in at least two ways: These are generally less polar substances and they may be preferentially absorbed by lipids, never reaching the sensitive site; alternatively the acylanilides may be hydrolysed enzymatically. If the activations of the thioureas and the inactivations of the acylanilides are indeed enzymic reactions, and if the enzymes involved are not universally distributed, there would seem to be some hope of discovering among the thioureas and acylanilides, herbicides with appreciable species specificity.

The herbicidal activity of the phenylcarbamates is not completely understood. It has long been known that these substances interfere with cell division, especially in grasses (4), and it was taken for granted that the plants were thereby killed. However, Moreland and Hill (11) have shown recently that most of the herbicidal phenylcarbamates also inhibit the Hill reaction and therefore they may inhibit photosynthesis. In fact both mechanisms may be operative and both may contribute to the death of the plant but the author is inclined to favor the original hypothesis for several reasons. Of these, the most compelling is the fact that isopropyl(2-chlorophenyl)-carbamate and isopropyl(2-methoxyphenol) carbamate are typical carbamate herbicides (17). From our work and the work of Moreland and Hill, it seems most improbable that such ortho-substituted anilides could be effective inhibitors of photosynthesis.

The manner in which the phenylureas, acylanilide, and triazine herbicides kill plants is not as obvious as one might think. Naturally, interference with the photosynthetic apparatus must ultimately lead to death of the plant through starvation. However, there is evidence that a more active process is involved since the phenylureas are toxic to sugar fed algae in the light but not in the dark (7). Since in this case there can be no question of starvation, it is presumed that deleterious oxidation processes accompany the accumulation of an oxidized photo-product, the photo-product which would be reduced, in the absence of inhibition, by water.

Summary

I. A large number of substances having the general formula R-NH-CX-R′ were obtained commercially or prepared and identified. These included acylanilides, acylamides, thioacylanilides, phenylureas, alkylureas, and phenylthioureas.

II. A study was made of the effectiveness with which these and miscellaneous other related compounds inhibit the reduction of ferricyanide by illuminated chloroplasts. A similar study was made of the inhibitory activity of several herbicidal triazines. The observations made possible certain generalizations:

A. Substitution of the hydrogen on the 3-, 4- or 5- positions of the benzene ring of the aniline derivatives by Cl, Br, CH_3O, or CH_3 increases the inhibitory effect. Substitution of the ortho position practically abolishes inhibition. Other parts of the molecule being equal, the inhibitory activity of the chloroanilides follows this sequence: 3,4-> 3,5- and 3- and 4- > 2,4,6- > unsubstituted and 2,4,5- > 2,5- and 2,3 > 2-. Para- and meta-nitro groups reduce activity slightly and the *p*-dimethylamino group reduces activity sharply.

B. The effects of modifying the acyl moiety of the anilides are too complex to classify. Steric considerations seem to be paramount since the chloro acids and their methyl analogs yield anilides of comparable potency. Polar groups reduce activity.

C. Chloracetyl-N-methylaniline, an anilide which lacks an imino hydrogen atom, is scarcely inhibitory.

D. Among the triazines, the substances with two imino hydrogens are more inhibitory than those with one.

III. The acylanilides, phenylureas, and triazines, like CMU, inhibit chloroplast reactions when ferricyanide or FMN is the electron acceptor but not when PMS is the acceptor. This probably means that the mechanism inhibited by all of these chemicals is the mechanism normally responsible for the oxidation of water to molecular oxygen.

IV. Attempts to relate inhibitor potency to the bonding tendency of the imino hydrogen were only partially successful; the role of hydrogen bonding in the mechanism of inhibition remains uncertain. Substitutions on the aniline moiety which favor hydrogen bond formation also increase the effectiveness of the inhibitor. However, modification of the acyl moiety did not reveal a similar correlation; the imino hydrogens of chloracetyl- and trichloracetyl-3,4-dichloroaniline form bonds with carbonyl oxygens to a very limited extent although both substances are excellent inhibitors. The same is true of the triazines.

Acknowledgments

This investigation would have been impossible without the able technical assistance of Mr. S. T. Bajura; I am much indebted to Mrs. Dorle Bongart for the numerous analyses. I also wish to express my gratitude to Dr. G. D. Thorn for his advice.

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OXIDATIVE ACTIVITY OF CYTOPLASMIC PARTICLES OF APPLES: KREBS CYCLE OXIDATIONS, OXIDATIVE PHOSPHORYLATION, & CYTOCHROMES[1]

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Previous studies of procedures for isolating apple mitochondria showed the importance of pH during homogenization of the tissues (12, 13). Virtually no mitochondrial activity could be detected in particles from neutral homogenates. Highest comparative rates of reduced diphosphopyridine nucleotide (DPNH) oxidase, DPNH- cytochrome c reductase, diaphorase, and cytochrome oxidase were recorded with particles from pH 9.9 homogenates. Maximal succinoxidase and succinic-cytochrome c reductase activities were found in particles from pH 8.4 homogenates. These particles were, therefore, characterized with respect to their electron transport chain activities. The purpose of the present study is to further characterize these apple particles and establish whether or not they can be identified as mitochondria. It will be shown that Krebs cycle activity, oxidative-phosphorylation, and cytochrome spectra, typical of other plant mitochondria, can be demonstrated with some of these particles. These data thus provide presumptive evidence for the presence of mitochondria in apple pulp tissue.

Materials & Methods

Rome Beauty apples [*Malus sylvestris* (Miller)] harvested in October 1959 and held in cold storage (2 C) during the winter and spring of 1959 to 1960 were used in these experiments. Cytoplasmic particles were isolated from peeled apples as previously described (13).

Krebs cycle activity was determined at 25 C by standard Warburg manometric techniques (16). No attempt was made to establish cofactor requirements for these systems. However, a mixture of cofactors, described in the appropriate table, was added routinely to each vessel.

Malate dehydrogenase activity was assayed by following oxidation of DPNH spectrophotometrically at 340 mμ in a system in which oxalacetate was reduced to malate, according to the method of Ochoa (6). Isocitrate dehydrogenase was determined by the increase in absorption at 340 mμ resulting from reduction of triphosphopyridine nucleotide (TPN) in a system in which isocitrate was oxidized. The method of assay was essentially that of Ochoa (6).

Krebs cycle reaction mixtures were chromatographed after a 4-hour incubation period. Reaction mixtures were pipetted into 2 volumes of absolute ethanol in 15-ml conical centrifuge tubes and the tubes placed in boiling water for 5 minutes. The denatured protein was precipitated by centrifugation and the supernatant reduced to about one milliliter in an evaporator. About 50 μl of each reaction mixture were spotted on Whatman No. 3 paper and chromatographed with 5 M butanol-formate (10).

Oxidative phosphorylation was carried out as previously described (11). Details of the reaction mixtures and the length of incubation are listed in the appropriate table.

Apple particles were prepared for spectrophotometric study by removing chlorophyll with 25 volumes of cold (−15 C) acetone, washing the precipitate with acetone, and resuspending in distilled water. The spectra were obtained with an instrument designed to record spectra of dense suspensions (4).

Low-temperature spectra were determined by immersing 2 ml of acetone-extracted particulate suspensions in a bath of liquid nitrogen. The suspensions were held in a cylindrical brass cell with a window as its base. This cylinder was lowered into a small Dewar flask, with an optical window base, containing liquid nitrogen. A frozen disk of 2 to 4 mm in thickness was obtained. The Dewar flask was placed directly over an end-window phototube for determination of spectra (14).

Reduction of cytochromes by succinate in the presence of hydroxyquinoline–N–oxime (HOQNO) was carried out by incubating with the suspensions for 5 minutes, before acetone extraction. After acetone extraction the particles were found to be inactivated, and further enzymatic oxidation or reduction could not be effected.

Nitrogen determinations were made by the method of Thompson and Morrison (15).

The data are the averages of at least three separate determinations. The range of variations from the mean was 10 to 20 % for Krebs cycle oxidations and oxidative-phosphorylation and 10 to 15 % in the spectraphotometric assays.

[1] Received April 21, 1961.

TABLE I

KREBS CYCLE ACTIVITY OF MITOCHONDRIA ISOLATED FROM HOMOGENATES OF DIFFERENT pH $Qo_2(N)$*

BUFFER**	pH OF HOMOGENATE	ENDOGENOUS	CITRATE	α-KETOGLUTARATE	SUCCINATE	MALATE	PYRUVATE
0.1 M K_2HPO_4							
0.1 M KH_2PO_4	6.5	6	14	23	19	2	4
0.2 M K_2HPO_4	7.2	8	18	18	36	11	14
0.05 M Tris	7.5	7	111	114	136	64	34
0.1 M Tris	8.4	21	140	235	352	175	106
0.2 M Tris	8 6	34	143	230	362	172	116
0.2 M Na_2CO_3	9.9	10	17	16	88	21	18

* Microliters oxygen consumed per hour per milligram nitrogen.

** Each buffer solution was 0.25 M with respect to sucrose and 0.01 M with respect to ethylenediamine tetraacetic acid.

Oxidative rates were determined in Warburg vessels shaken at 120 strokes per minute for 1 hour at 25 C, after equilibration for 10 minutes. Center wells contained 0.2 ml 10 % KOH with filter paper wicks. Each vessel contained 2 μmoles adenosine triphosphate (ATP), 10 μmoles glucose, 18 μmoles Mg^{++}, 40 μmoles $PO_4^{=}$ 0.3 μmoles diphosphopyridine nucleotide (DPN), 0.06 μmoles cytochrome c, 1 mg hexokinase, 1 ml 2 % yeast concentrate (Sigma), and 30 μmoles substrate acid. One milliliter of mitochondrial suspension, with a nitrogen content varying from 300 μg to 900 μg depending on the homogenate from which the particles were isolated (13), was added to each vessel. The pyruvate vessels contained 4.5 μmoles malate as primer acid. Total volume in the incubation mixture was 3 ml.

Additional details are described in the text and legends of the tables and figures.

RESULTS

KREBS CYCLE OXIDATIONS: Rates of oxidation of Krebs cycle substrates by particles from homogenates of different pH are shown in table I. Every substrate examined showed maximal activity with particles from homogenates of pH 8.4 and 8.6 (table I). Very little activity was obtained with particles from homogenates of pH 6.5, 7.2, and 9.9.

Particles from tris-homogenates of pH 7.5 exhibited considerably greater oxidative rates than those from phosphate-homogenates of pH 7.2. Since this small difference in pH could hardly account for the wide changes in oxidative rate, a deleterious effect of phosphate buffer is indicated. Inhibitory effects of phosphate on mitochondrial activity have been noted previously (8).

Of all substrates tested, succinate gave the best oxidative rates with practically all particles, regardless of pH of the initial homogenate. It is possible that the succinoxidase system normally has a much higher level of activity than other Krebs cycle enzymes. It is more likely, however, that the succinoxidase system is much better preserved in these particles (except for those from pH 6.5 homogenates) than are other Krebs acid oxidases. This seems to be especially true in the particles from homogenates of pH 9.9.

Highest rates for malate dehydrogenase activity were obtained with particles from homogenates of pH 9.9 and lowest rates with those isolated from neutral buffers (table II). However, isocitrate dehydrogenase activity was approximately equal in particulate preparations from homogenates of pH 7.2, 8.4, and 9.9. On the basis of these data, there appeared to be no correlation between rates of dehydrogenase activities and oxidase activities.

TABLE II

ACTIVITY OF MALATE DEHYDROGENASE & ISOCITRATE DEHYDROGENASE IN MITOCHONDRIA ISOLATED FROM HOMOGENATES OF DIFFERENT pH

pH OF HOMOGENATE*	SPECIFIC ACTIVITY**	
	MALIC DEHYDROGENASE***	ISOCITRIC DEHYDROGENASE†
7.2	0.72	0.07
8.6	1.74	0.07
9.9	4.38	0.10

* Homogenates are same as described in table I.

** Specific activity is in terms of μmoles of DPNH oxidized or TPN reduced per minute per mg nitrogen. Rates were calculated from the absorption coefficients (13).

*** Malate dehydrogenase was assayed in a mixture of 75 μmoles glycylglycine buffer pH 7.4, 0.15 μmoles DPNH, 0.76 μmoles oxalacetic acid, and water to a volume of 2.95 ml. The reaction was started by adding 0.05 ml of mitochondrial suspension. Other details are described in text.

† Isocitrate dehydrogenase was assayed in a mixture of 75 μmoles glycylglycine pH 7.4, 1.8 μmoles $MnCl_2$, 0.13 μmoles TPN, 0.6 μmoles monopotassium isocitrate and water to a volume of 2.95 ml. Reaction was started with 0.05 ml mitochondrial suspension. Additional details are described in text.

CHROMOTOGRAPHY OF REACTION MIXTURES: Additional evidence for the very rapid oxidation of succinate by apple particles was seen in chromatograms of reaction mixtures from Warburg vessels. The chromatogram in figure 1 shows the conversion of citrate, α-keto-glutarate, succinate, malate, and fumarate to succeeding acids in the Krebs cycle. Succinate, however, does not appear on the chromatogram unless its oxidation is inhibited by malonate. This indicates that succinate is oxidized more rapidly than any of the other acids. Therefore, the oxidation observed by Warburg manometry, with all substrates, is due for the most part to the succinoxidase system. In figure 1 are the results obtained with particles from homogenates of pH 8.4. Similar results were also obtained with particles from homogenates of pH 9.9, after incubation for longer periods.

No chromatographic spots were obtained for pyruvate or any intermediate expected from its oxidation, in the presence or absence of malonate (fig 1). Generally pyruvate shows up very poorly in these chromatographic systems. Since pyruvate caused oxygen uptake in the Warburg vessels, spots for Krebs cycle intermediates should have appeared, especially in the presence of a malonate block. However, the low rate of pyruvate oxidation makes it likely that the concentrations of intermediates were too low to be detected by the techniques used, even in the presence of malonate. Most of the oxidation in these systems could have been derived from malate used as a primer acid.

OXIDATIVE PHOSPHORYLATION: A more critical comparison of particles from pH-9.9 and pH-8.4 homogenates, was obtained from data on oxidative-phosphorylation. The greater activity of the particles from tris-homogenates of pH 8.6 is clearly evident (table III). Some measure of phosphorylation was achieved with these particles, but particles from homogenates of pH 9.9 gave no oxidative-phosphorylation. On this basis, particles from the tris-homogenates appear to be more akin to those in the intact cell. However, the phosphorylation was quite low compared with that of other plant mitochondria. This indicates that the phosphorylation steps were not well preserved in these isolated particles.

SPECTRA OF PARTICLES: The relatively high concentration of chlorophyll and low concentration of cytochromes made it difficult to observe spectra of cytochromes in apple particles (13). However, after

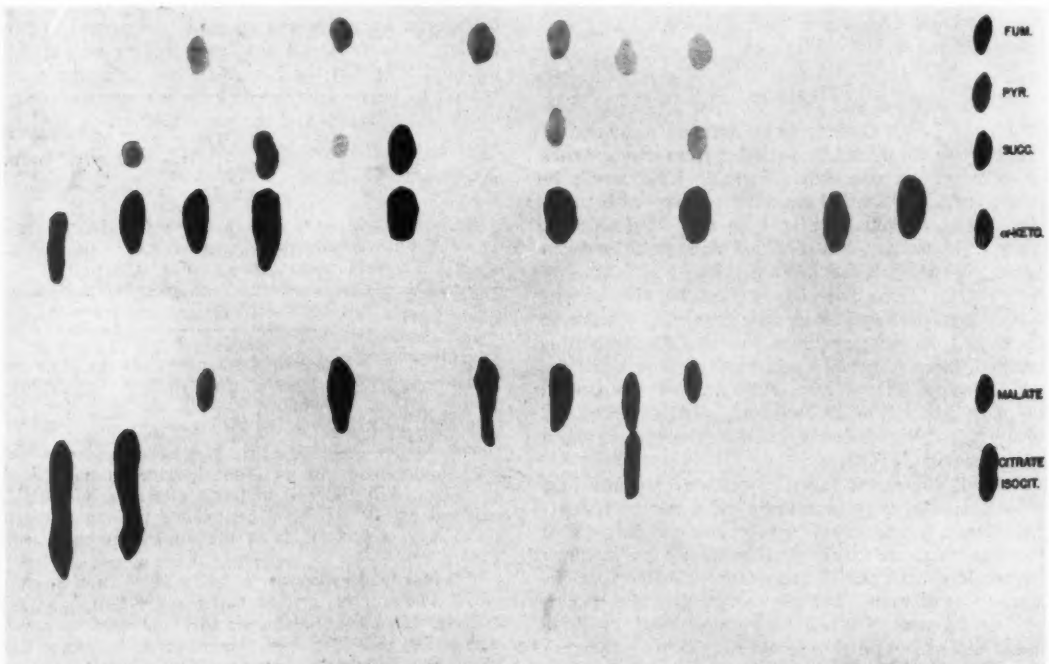


FIG. 1. Chromatogram of reaction mixture in Warburg vessels. Particles were from homogenate of pH 8.4. Content of the reaction mixture is described in table I. Malonate, 3 μmoles, was used in the inhibited systems. Approximately 200 μg of each known acid were used as marker. Other details of methodology are described in the text.

TABLE III

OXIDATIVE-PHOSPHORYLATION BY CYTOPLASMIC PARTICLES FROM APPLES*

pH OF HOMOGENATE*	SUBSTRATE	μ ATOMS P	μ ATOMS O	P/O***
8.6	Endog.	+ 1.7	2.1	...
"	Succinate	13.6	25.2	0.5
"	α-Keto	14.5	16.7	0.9
"	α-Keto + Malonate	11.6	8.8	1.3
9.9	Succinate	+ 3.1	0.4	...
"	Endog.	+ 1.2	6.2	...
"	α-Keto + Malonate	+ 3.5	2.5	...

* In these experiments oxygen was determined during 2 hours of incubation and phosphorus was determined before and after the incubation period.

** Buffers and homogenates are described in table I.

*** Reaction mixtures contained additions listed in table I. In addition 3 μmoles of malonate were added to vessels where indicated. Further details of methodology were carried out as previously reported (11).

extraction of chlorophyll with cold (−15 C) acetone and addition of calcium carbonate to increase the optical path length (4), it was possible to obtain spectra of cytochromes in suspensions reduced with dithionite (fig 2). Absorptions at about 530 mμ, 560 mμ, and 600 mμ were observed, in addition to an absorption at 640 mμ which was due to residual chlorophyll (fig 2). The peaks at 530 and 560 mμ were attributed to the β and α bands of cytochrome b and the small absorption at 600 mμ to cytochrome a plus a_3 (5). Two interesting aspects in these spectra are the absence of cytochrome c and the very large absorption of cytochrome b relative to the absorption of a plus a_3.

The absorption bands were more clearly defined by studying spectra of acetone-extracted particles at liquid-nitrogen temperatures (−195 C). At −195° the cytochrome spectra are intensified and given greater detail as shown by a number of workers with plant mitochondria (9, 2, 3, 1).

Low-temperature spectra of all particles, after reduction with dithionite, showed an absorption band around 598 to 599 mμ (figs 3, 4, 5, 6). This is due to cytochrome a + a_3, the cytochrome oxidase component which appears to be prominent in all the particles studied. The next absorption band common to all particles has a peak at about 556 to 558 mμ. This is related to the large 560 mμ peak seen at room temperatures, the most intense band in apple particles, and is attributed to cytochrome b. There is also an absorption band at 527 to 528 mμ shown in all these spectra. This is undoubtedly the β band of cytochrome b and corresponds to the 530 mμ band observed at room temperatures. Characteristically absorption bands are shifted to shorter wavelengths at liquid nitrogen temperatures (9). The ratio of cytochrome b to a + a_3 as judged from the absorptions at 556 to 558 mμ and 598 to 599 mμ appeared to be about 2. This is considerably less than indicated in the spectra obtained at room temperature (fig 2).

The additional absorption bands in these spectra (figs 3, 4, 5, 6) are less distinct and are not peaks but shoulders on the 556 to 558 mμ absorption band. These are best seen in figure 4 curves 2 and 4. Curve 2 shows a broad shoulder from 549 to 552 mμ with an indentation at 549 mμ. Curve 4 shows a bulge at 549 mμ. Particles from homogenates of pH 9.8, reduced with succinate in the presence of HOQNO, showed a broad absorption band from 548 to 552 mμ (fig 3 curve 2). A prominent shoulder at 552 mμ was also observed in curve 3 of this figure (fig 3). In figure 5 there is a shoulder at 552 mμ and in figure 6 there is a small absorption band at 548 mμ.

Taken together these data (figs 3, 4, 5, 6) give evidence for absorption bands at 548 to 549 mμ and 552 mμ. These absorption bands are best seen in the particles from homogenates of pH 8.4 (fig 4) which

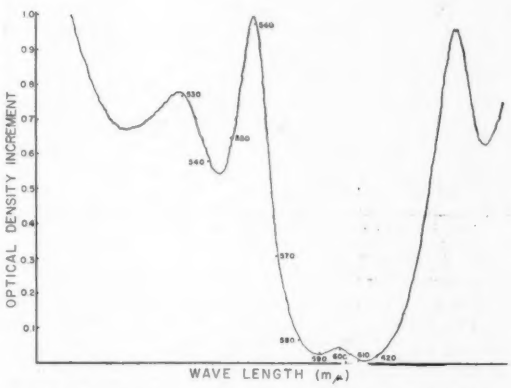
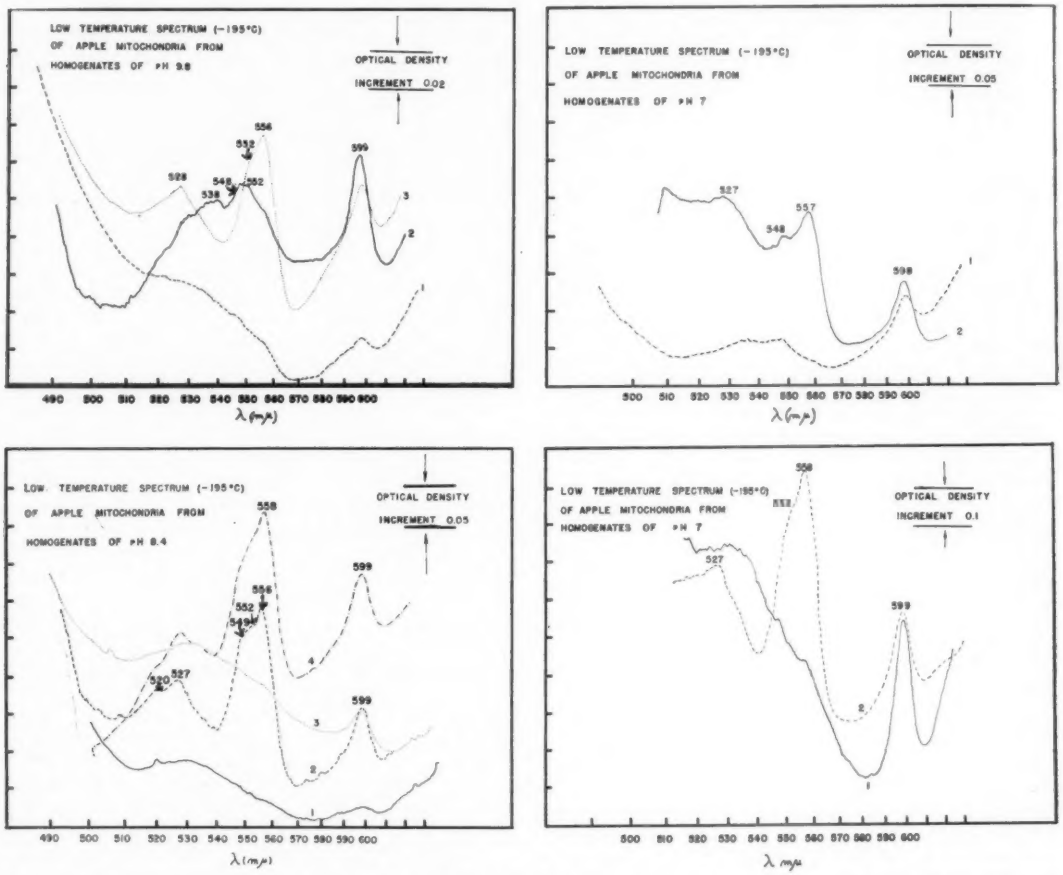


FIG. 2. Spectrum of concentrated preparation of cytoplasmic particles from apples after extraction with cold (−15 C) acetone, addition of calcium carbonate, and reduction with dithionite. The light path was 1.5 cm with approximately threefold intensification due to carbonate (4). These concentrated preparations from homogenates of pH 9.9 had a nitrogen content of approximately 800 mg per ml.

are the most active particles. The 552 mμ absorption may be analogous to the peaks observed in this region in low temperature spectra of plant mitochondria by Bendall (2), Bonner (3), and Baker and Lieberman (1).

The absorption band at 548 to 549 mμ is most probably cytochrome c. The small absorption band seen at 520 mμ in figure 4 curve 2 is in the correct region for the β band of cytochrome c, and therefore lends additional support to the presence of cytochrome c in these particles. The presence of cytochrome c in these particles is also indicated from data on enzymatic activity (13).

Addition of succinate and HOQNO to the particles increased the intensity of the bands at 598 to 599 mμ in all the particles. However, succinate and HOQNO had little effect on increasing absorption in the 548 to 560 mμ region. Addition of dithionite to the particles reduced with succinate in the presence of HOQNO, quickly brought out the absorption band



Low temperature spectra (−195 C) of apple particles from homogenates of different pH. The particles were extracted with cold acetone to remove chlorophyll as described in the text. Two milliliters of preparation were used.

FIG. 3 (*top left*). Spectrum of particles from homogenates of pH 9.8. Nitrogen content of preparation was about 250 μg per ml. Curve 1—non-reduced particles; curve 2—particles reduced with 200 μmoles succinate in presence of 0.1 mg HOQNO before acetone extraction; curve 3—particles reduced with dithionite.

FIG. 4 (*bottom left*). Spectrum of particles from homogenates of pH 8.4. Nitrogen content was about 550 μg per ml. Curve 1—non-reduced particles; curve 2—particles reduced with dithionite; curve 3—particles reduced with 200 μmoles succinate in presence of 0.1 mg HOQNO; curve 4—succinate-reduced particles (with HOQNO) further reduced with dithionite.

FIG. 5 (*top right*). Spectrum of particles from homogenates of pH 7.0. Nitrogen content about 900 μg per ml. Curve 1—non-reduced particles; curve 2—particles reduced with dithionite.

FIG. 6 (*bottom right*). Spectrum of particles from homogenates of pH 7.0. Nitrogen content about 900 μg per ml. Curve 1—particles reduced with 200 μmoles succinate in presence of 0.1 mg HOQNO; curve 2—succinate reduced particles (with HOQNO) further reduced with dithionite.

at 557 to 558 mμ and the broad shoulder at 548 to 552 mμ (figs 4, 6). The important point shown by these low-temperature spectra is that the detailed cytochrome spectra in the apple particle are much like those observed in other plant mitochondria.

Discussion

There has been some question as to whether fruit tissues, such as apple pulp, which show high rates of phenolase activity, also possess typical mitochondrial patterns of respiratory activity. The present report and data from a previous study (13) provide evidence for the presence of mitochondrial activity in particulate preparations isolated from apple homogenates of pH 8.4 to 8.6. These particles possess electron transfer chain activity, Krebs cycle activity, ability to carry out oxidative-phosphorylation, and show most of the cytochrome spectra observed in other plant mitochondria. Particles isolated from neutral buffers showed very little mitochondrial activity, but did exhibit spectra indicating the presence of cytochromes. Particles from homogenates of pH 9.9 were like mitochondria in some of their reactions. They showed no oxidative-phosphorylation and their Krebs cycle activity was very low. However, they exhibited highest comparative rates of DPNH-oxidase, DPNH-cytochrome c reductase, diaphorase, and cytochrome oxidase activity (13).

The particles from neutral and very alkaline buffers (pH 9.9) must represent mitochondria that have been damaged in different ways and in different degree. Particles from neutral buffers were probably inactivated by adsorption of deleterious proteins during the isolation procedure. This appears likely from the high nitrogen values of these preparations (13). Particles from very alkaline homogenates (pH 9.9) seem to suffer from the reverse phenomenon. It appears that fragmentation, partial solubilization, or another type of disruption of the mitochondria occurred in these homogenates. This is presumed from the very low nitrogen values of the preparations.

The highest comparative rates of electron transport chain activity, except for succinate-cytochrome c reductase, and the highest rates of malate dehydrogenase activity, displayed by the particles from homogenates of pH 9.9, may be explained by the so-called opening phenomenon described by Green (7). Succinoxidase and succinate-cytochrome c reductase are not stimulated in these particles because these systems are more resistant to alkaline buffers and therefore are not opened as much as the other components of the electron chain. Evidence for better preservation of the succinoxidase system in particles from pH 9.9-homogenates is shown by the much higher rate of oxidation of succinate compared to other Krebs acids and by the lack of accumulation of succinate on the chromatograms. Release of phosphate from pH 9.9-particles, in the oxidative phosphorylation experiments, provides evidence for adenosine triphosphatase activity. This is an additional indication of injury to the mitochondria by the alkaline buffers causing, in this case, damage to the phosphorylating system.

The opening of malate dehydrogenase by alkaline homogenates (pH 9.9) increased its activity considerably. However, the increased activity is the reverse of that necessary to provide oxygen uptake. The stimulated reaction goes from oxalacetate to malate consuming DPNH rather than producing it. This may be the explanation for the low rates of oxygen uptake with Krebs acids by these particles despite its very active electron transport chain.

Low-temperature spectra of the particles revealed absorption bands at around 549 mμ and 552 mμ, which were not seen in the spectra at room temperatures. At room temperature the intense band at 560 mμ overshadows the smaller bands and masks their presence. Even at liquid-nitrogen temperatures the bands at 549 mμ and 552 mμ appear as a wide shoulder to the large 556 to 558 mμ band. This is due to the proximity of the 549 and 552 mμ bands and to the large pull to longer wavelengths by the strong 556 to 558 mμ absorption band. Nevertheless, absorption bands at 549 mμ and 552 mμ are clearly visible. These bands are best seen in the particles (pH 8.4) that are most active and show all activities of mitochondria.

Identification of cytochrome oxidase and other electron transfer chain components enzymatically and spectrally, and the small bands at 549 mμ visible in the low-temperature spectra, serve to identify cytochrome c as a component of the apple mitochondria. Since cytochrome c is the most soluble cytochrome it is very probable that much of it was removed from the particles by solubilization during the preparative procedure. This may account for the difficulty in observing the spectrum of cytochrome c in apple particles.

The absorption band at 552 mμ seen at low temperatures, may be analogous to the band seen at 552 to 554 mμ in other plant mitochondria at low temperatures (2, 3). There was no band, in the spectra of apple particles at low temperature, to correspond to the band at 562 mμ seen by Bonner (3) in spectra of a number of plant mitochondria, and by Bendall (2) and Baker and Lieberman (1) in spectra of sweetpotato mitochondria at low temperature. The purpose of the addition of HOQNO and succinate was to bring out this band, as was done with sweetpotato mitochondria. This was not observed with apple particles. Since the 562 mμ absorption band is the weakest band in this region, its concentration may be too low for observation, even after dithionite treatment. It is also possible that apple mitochondria do not have this component. In other respects, except for quantitative aspects, apple mitochondria from homogenates of pH 8.4 to 8.6 appear similar to other plant mitochondria.

Summary

Further comparative studies were completed of apple particles isolated from neutral and alkaline homogenates. Particles from homogenates of pH 8.4 to 8.6 showed highest rates of Krebs cycle oxida-

tion, oxidative phosphorylation, and clearest cytochrome spectra. These particles, therefore, contain a high percentage of mitochondria.

Particles from approximately neutral homogenates (pH 6.5–7.2) showed very little mitochondrial activity. Particles from homogenates of pH 9.9 exhibited good succinoxidase activity, highest comparative malate dehydrogenase activity, but no oxidative phosphorylation. Rates of isocitrate dehydrogenase activity were the same in all these particles.

Low-temperature spectra (−195 C) of the particles revealed a band at 599 mμ identified as cytochrome a plus a_3, a band at 556 to 558 mμ and 527 to 528 mμ identified as the α and β bands of cytochrome b, and weaker bands at 548 to 549 mμ and 552 mμ. The 548 to 549 mμ band was recognized as cytochrome c and the 552 mμ band was assumed to be analogous to a similar band observed at low temperature in other plant mitochondria. These latter bands were most conspicuous in particles from homogenates of pH 8.4.

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DEOXYRIBONUCLEIC ACID OF COTTON[1]

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The deoxyribonucleic acid (DNA) composition of microorganisms has been studied extensively and the vast data obtained form the basis for the concept of chemical specificity of nucleic acids in general (1). In contrast, the DNA composition of higher plants has received relatively little attention (11, 14, 15, 16). This applies particularly to studies, e.g., those on wheat (11, 16), in which isolation and purification of the DNA have preceded determination of the constituent purine and pyrimidine bases.

In this paper we report the isolation and composition of DNA from nine genotypes of the genus Gossypium, in which two species, four races, and five varieties are represented. Although we give major emphasis to cotton, we also include limited data on the DNA composition of corn, peanut, sunflower, and wheat for comparison.

MATERIALS & METHODS

DESCRIPTION OF COTTON GENOTYPES[2]: Paymaster 54B, Deltapine 15, and Stoneville 2B are cultivated varieties of American Upland cotton, species *Gossypium hirsutum* L. The Red Empire stock used in this study, while qualifying as a variety of *G. hirsutum*, carries a simply inherited gene for red leaves which distinguishes it from the usual green leaf types. Latifolium, punctatum, richmondi, and palmeri are primitive or noncommercial types of *G. hirsutum* which have been identified by a subclassification of the species into geographical races. Only one stock of *G. barbadense*, an agricultural variety named Pima S–1, was used. Characteristically, cultivated vareties of this species have smaller bolls and longer, stronger, and finer fibers than those of *G. hirsutum*.

ISOLATION OF Na-DNA FROM COTTON: All operations, except as noted, were conducted at 0 to 10 C.

1. Freshly-decorticated cotton embryos, 300 g, were homogenized in a high-speed mixer with 500 ml of 95 % ethanol for 5 minutes. After adding another 500 ml of ethanol and 1 liter of ether, the mixture was allowed to stand for 2 hours and then the solvent was removed by suction. The residue was ground to 20-mesh fineness in a small Wiley mill, suspended in 2 liters of acetone, and stored for 24 hours with occasional stirring.

2. The acetone was removed by suction and the residue washed with 100 ml portions of acetone until the washings produced no appreciable precipitate upon the addition of an equal volume of water. Air-dry weight of the defatted residue was approximately 190 g. A suspension of the residue in 1 liter of 0.1 M NaCl was stirred vigorously for 3 hours with a heavy duty stirrer (paddle-type propeller) and centrifuged at 2,520 × *g* for 15 minutes. After discarding the supernatant, the residue was washed and centrifuged 12 times in a similar manner with 0.1 M NaCl.

3. The washed residue was next suspended in 500 ml of 0.9 % NaCl and 45 ml of Duponal (10) solution (5 g of purified Duponal, essentially sodium dodecyl sulfate, in 100 ml of 45 % ethanol) were slowly added while stirring. The mixture was stirred vigorously for 3 hours with a heavy-duty stirrer.

4. The mixture was then centrifuged for 3 hours at 2,520 × *g* and sufficient NaCl was added to the decanted supernatant to bring the concentration of NaCl up to 1 M. An equal volume of 95 % ethanol was added with stirring to this solution and the resultant precipitate of crude Na-DNA, which existed in both fibrous and granular form, was collected by centrifugation and washed once with 80 % ethanol.

5. The crude Na-DNA was dissolved in 300 ml of 0.14 M NaCl by stirring for 2 hours at 25 C. Duponal solution, 27 ml, was added and the mixture was stirred for 1 hour. After sufficient NaCl was added to make the NaCl concentration 1 M, the solution was centrifuged at 21,600 × *g* for 1 hour at 25 C. After adding an equal volume of 95 % ethanol to the decanted supernatant, the Na-DNA, which separated as a fibrous mass, was collected by spooling. The Na-DNA was washed successively with 80 % and 95 % ethanol, acetone, and air-dried. The Na-DNA (approximately 500 mg) at this stage still contained a small amount of protein, some free nucleotides, and

[1] Received May 2, 1961.

[2] For detailed information on the origin and classification of the species of Gossypium and the races of *G. hirsutum* refer to: A: J. B. Hutchison, Heredity 5: 161–193 (1951) and B: The Evolution of Gossypium and the Differentiation of the Cultivated Cottons. J. B. Hutchison, R. A. Silow, and S. G. Stephens, Oxford University Press, London.

RNA (ribonucleic acid), the major contaminant. The next step removed all but the RNA impurity.

6. The Na-DNA from step 5 was dissolved in 300 ml of 25 % ethanol by stirring for 2 hours at 25 C. The solution was made 1 M with NaCl and centrifuged for 1 hour at 25,000 × *g*. The Na-DNA was precipitated from the supernatant with one-half volume of 95 % ethanol, washed, and dried as in step 5. Air-dried weight of the fibers was about 350 mg.

7. The Na-DNA from step 6 was dissolved in 300 ml of 0.14 M NaCl and stirred for 2 hours with 15 ml of washed Norit A, which selectively adsorbs RNA (17). The solution was then clarified by centrifuging for 2 hours at 28,700 × *g*, brought to 1 M with NaCl, and the Na-DNA precipitated by adding 1 volume of 95 % ethanol. The yield of fibers, washed with alcohol and acetone until free of chlorides, and air-dried, was approximately 150 mg. This weight represents about 50 % of the total DNA content of the embryos (7).

Isolation of Na-DNA From the Other Genera: Na-DNA of corn (germ), peanut (embryo), and sunflower (embryo) was isolated and purified by the method described for cotton. Na-DNA of wheat (germ) was obtained by the method of Lipshitz and Chargaff (11).

Analytical: Hydrolysis of the Na-DNA isolates to the free purine and pyrimidine bases and their chromatographic separation and determination followed closely the procedure outlined by Lipshitz and Chargaff (11). Four hydrolysates of each isolate were used for determining the bases, which are expressed as moles per 100 gram-atoms of phosphorus. Using four spottings of 0.06 ml per hydrolysate, the standard deviation of each free base ranged from 0.10 to 0.22 mole per 100 gram-atoms of phosphorus. Phosphorus was determined by the Fiske-Subbarow methed (8).

Results & Discussion

The method used for isolating DNA from cotton, which differs in certain essentials from that used by Lipshitz and Chargaff (11) on wheat germ, resulted from unsuccessful attempts to apply their method to cotton. The method for cotton was applicable also to corn, peanut, and sunflower, but it was not tested on wheat germ and the method of Lipshitz and Chargaff was not tried on the other species.

In general all the Na-DNA isolates met the following requirements for satisfactory products (4): A: absence of proteins (biuret) and ribonucleic acid (uracil); B: a phosphorus content close to 9.2 %; C: a maximum absorption in the ultraviolet at 257 to 261

Table I

Composition of Total Deoxyribonucleates of Cotton*

	Moles/100g-atoms P**						Molar ratios		
	A	T	5MC	C	G	Actual recovery	A/T	Pu/Py	G/C+5MC
G. hirsutum									
Race:									
Latifolium	32.7 (0.05)***	33.0 (0.07)	4.6 (0.06)	12.8 (0.05)	16.9 (0.11)	94.7	0.99	0.98	0.97
Punctatum	32.8 (0.04)	33.0 (0.08)	4.6 (0.08)	12.7 (0.05)	16.9 (0.08)	93.3	0.99	0.99	0.98
Richmondi	32.8 (0.08)	33.0 (0.25)	4.6 (0.08)	12.6 (0.03)	16.9 (0.05)	95.4	0.99	0.99	0.98
Palmeri	32.7 (0.13)	32.8 (0.05)	4.7 (0.06)	12.8 (0.08)	17.1 (0.11)	91.4	1.00	0.99	0.98
Varieties:									
Red Empire	32.8 (0.14)	33.0 (0.06)	4.6 (0.05)	12.7 (0.05)	16.8 (0.25)	96.3	0.99	0.99	0.97
Paymaster 54B	32.8 (0.06)	32.8 (0.05)	4.6 (0.05)	12.8 (0.03)	17.1 (0.07)	98.4	1.00	0.99	0.98
Deltapine 15†	32.8 (0.05)	33.0 (0.10)	4.5 (0.05)	12.8 (0.03)	16.9 (0.06)	92.6	0.99	0.99	0.98
Stoneville 2B†	32.7 (0.03)	32.8 (0.05)	4.4 (0.03)	12.9 (0.03)	17.1 (0.03)	90.5	1.00	0.99	0.97
G. barbadense									
Pima S-1 (Var.)	32.9 (0.03)	33.1 (0.04)	4.6 (0.03)	12.5 (0.03)	16.8 (0.05)	94.9	0.99	0.99	0.98

* Abbreviations: A, adenine; T, thymine; 5MC, 5-methyl cytosine; C, cytosine; G, guanine; Pu, purines; Py, pyrimidines.

** Mean proportion of each constituent corrected for a 100 % recovery. Actual recovery is given in the seventh column.

*** The standard error of four hydrolysates.

† Double haploid.

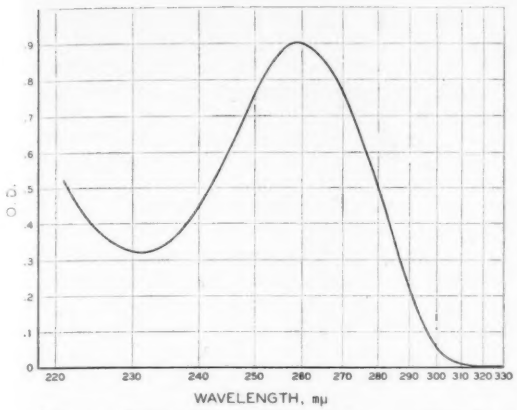

FIG. 1. Ultraviolet absorption curve of cotton Na-DNA (0.0046 %). Curve reproduced from that drawn by a DK-2 spectrophotometer.

mμ (fig 1); D: molar adsorptivity, relative to phosphorus, around 6,600 at 260 mμ, and E: a fibrous character (fig 2).

FIG. 2. Suspension of cotton Na-DNA in 95 % ethanol, showing characteristic fibrous nature of all DNA's.

The composition of the DNA's from the nine cotton genotypes, together with a summary of some of the molar relationships, is given in table I. The ratios A/T, Pu/Py, and G/C+5MC satisfactorily reflect the unity relationships, first noted by Chargaff (5), which are characteristic of all DNA's. Note, however, that the DNA's of the nine types of cotton differed little from one another in base content. Adenine varied from 32.7 to 32.9 mole percent, thymine 32.8 to 33.1, 5-methyl-cytosine 4.4 to 4.7, cytosine 12.5 to 12.9, and guanine 16.8 to 17.1. In addition to the ratios of table I, the DNA bases of each genotype also were expressed as ratios of A: A+T/G+C+5MC and B: C/5MC, and the differences within each were evaluated statistically. The former ratio, while adequate in showing the overall composition, does not reflect differences that may exist in the degree of 5-methylcytosine substitution for cytosine; hence, use of the C/5MC ratio.

TABLE IIA

STATISTICAL ANALYSIS OF C/5MC RATIOS IN COTTON DNA ISOLATES. ANALYSIS OF VARIANCE OF C/5MC RATIOS*

VARIANCE	° OF FREEDOM	MEAN SQUARE	F VALUE
Total	35		
Isolates	8	0.01685	4.39**
Error	27	0.00384	

* Four for each of the nine DNA isolates.
** 3.26 needed at 0.01 level.

Preliminary to statistical evaluation, application of the Bartlett test (13) indicated that the variance within each of the ratios A+T/G+C+5MC and C/5MC from the nine cottons could be pooled for an analysis of variance. Results from the latter showed that differences in the DNA's exhibited by the A+T/G+C+5MC ratios were insignificant whereas significance was found among the C/5MC ratios (table II).

TABLE IIB

SEPARATION OF C/5MC MEANS FROM EACH ISOLATE BY DUNCAN MULTIPLE RANGE TEST

ISOLATES	MEANS			
Stoneville 2B	2.91	a**		
Deltapine 15	2.84	a	b*	
Punctatum	2.78		b	c
Latifolium	2.78		b	c
Richmondi	2.74			c
Paymaster 54B	2.74			c
Red Empire	2.73			c
Pima S-1	2.73			c
Palmeri	2.71			c

* P 0.05
** P 0.01

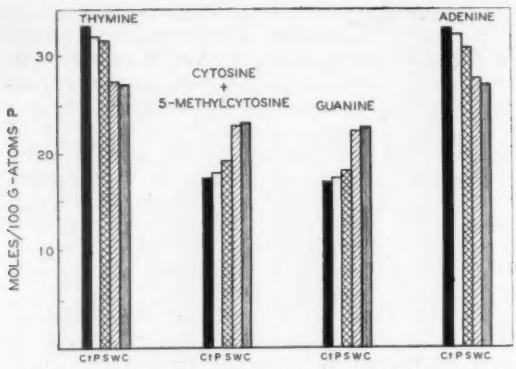

FIG. 3. Proportions of purine and pyrimidine bases in the DNA's of cotton (Ct.), peanut (P), sunflower (S), wheat (W), and corn (C).

The C/5MC ratios of Stoneville 2B and Deltapine 15 varieties (both double haploids), while not differing from each other, were significantly higher than those of the other genotypes except that Deltapine 15 did not differ significantly from the races latifolium and punctatum. Stated otherwise, the DNA of Stoneville 2B and, to a lesser extent, that of Deltapine 15, appeared to contain fewer 5-methylcytosine-guanine combinations than that of the other genotypes. What bearing, if any, the double haploid factor could have on this finding is not apparent at this time.

Turning from the cotton genotypes, among which there was no significant difference in the overall DNA compositions, to the other genera, certain species differences are indicated (table III). The DNA composition index, A+T/C+5MC+G, ranges from 1.17 for corn to 1.92 for cotton (average of all values in table I). Moreover, on the basis of plant classification, the dicots (cotton, peanut, & sunflower) have a higher index than the monocots (corn & wheat). A comparison of the proportions of purine and pyrimidine bases in the individual DNA's (fig 3) shows that adenine and thymine contents decrease and cytosine+5—methylcytosine and guanine increase in the order: cotton, peanut, sunflower, wheat, and corn. Examination of the data in table III shows that the percentage contribution of 5-methylcytosine to its sum with cytosine was substantial; it varied from 27 % in cotton to 37 % in sunflower. All the DNA's are of the AT type (adenine & thymine predominating).

After the present study was started, Russian workers (14, 15) published data on the DNA compositions (determined directly in the tissue without prior isolation) of some 26 plant species, including corn, peanut, sunflower, and wheat. Analytical agreement between the two sets of data is only fair, and of greater interest is that they found only small differences between representatives of widely different systematic groups. The composition index of the 26 species varied within the narrow range of 1.03 to 1.61. In some instances, like our data on corn and wheat (table III) and the two species of cotton (table I), almost identical compositions were found. In contrast, the DNA composition index of microorganisms has been found A: to vary over the wide range of 0.35 to 2.7, depending upon the species to which they belong, and B: to be species specific (1). However, it would appear that current data on the higher plants are not yet sufficiently numerous for any conclusion on the relationship between species and DNA composition.

Although the main interest of the results discussed herein centered on the overall composition of DNA from several higher plant sources, it is generally recognized, as expressed by Hotchkiss (9): "that different arrangements of bases in a nucleic acid chain —with or without a difference in overall composition —provide the means by which different specific molecules are constructed".

SUMMARY

I. We isolated the DNA's of nine cotton geno-

TABLE III

COMPOSITION OF TOTAL DEOXYRIBONUCLEATES OF COTTON, CORN, PEANUT, SUNFLOWER, & WHEAT*

	MOLES/100 g-ATOMS P**						MOLAR RATIOS			
	A	T	5MC	C	G	ACTUAL RECOVERY	A/T	Pu/Py	G/C+5MC	A+T / C+5MC+G
Cotton***	32.8	32.9	4.6	12.7	16.9	94.2	1.00	0.99	0.98	1.92
Corn	26.8	27.2	6.2	17.0	22.8	89.3	0.98	0.98	0.98	1.17
Peanut	32.1	32.2	5.7	12.3	17.6	93.2	1.00	0.99	0.98	1.81
Sunflower	30.8	31.6	7.1	12.0	18.5	87.8	0.97	0.97	0.97	1.63
Wheat	27.2	27.4	6.2	16.6	22.6	97.0	0.99	0.99	0.99	1.20

* Abbreviations: A, adenine; T, thymine; 5MC, 5-methylcytosine; C, cytosine; G, guanine; Pu, purines; Py, pyrimidines.

** Mean proportions of each constituent corrected for a 100 % recovery. Actual recovery given in the seventh column.

*** Average of all values in table I.

types and of corn, peanut, sunflower, and wheat, and determined and compared the base constituents.

II. The overall DNA compositions of the various cottons did not differ significantly from one another. However, the DNA's of the two double haploid varieties, as indicated by their cytosine/5-methylcytosine ratios, appeared to contain fewer 5-methylcytosine-guanine combinations than those of the ordinary genetic types.

III. Adenine and thymine contents decreased and cytosine plus 5-methylcytosine and guanine increased in the order: cotton, peanut, sunflower, wheat, and corn. All of the DNA's were of the adenine-thymine type.

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PHYSIOLOGICAL EFFECTS & DEGRADATION OF *S,S,S*-TRIBUTYLPHOSPHOROTRITHIOATE BY COTTON LEAVES AS SHOWN BY THE P^{32} & S^{35} LABELED COMPOUNDS [1]

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The first organic thiophosphorus compound reported to induce and accelerate abscission was *S,S,S*-tributylphosphorotrithioate (6, 14). Goyette (5) tested homologous trialkyl phosphorotrithioites and the corresponding trialkyl phosphates for abscission-inducing activity in the greenhouse and noted that only the former were active. Hall et al. (paper presented at the 134th National Meeting Am. Chem. Soc. Chicago, 1958, by W. C. Hall, F. R. H. Katterman, C. S. Miller, & F. A. Herrero) studied a wide array of phosphorus and sulfur containing compounds for abscission properties and confirmed Goyette's findings. Both groups of workers (5, & Hall et al.) concluded that the sulfur moiety was responsible for the defoliation response.

Our study is primarily concerned with the effect of this defoliant on respiration and on the -SH groups of water soluble proteins in the leaf, since both have been shown to be influenced by other defoliants (7, 10). In addition, we studied the rate and manner of degradation of this defoliant in the cotton leaf before and during abscission.

Methods & Materials

Preliminary results indicated that in vivo degradation of the trithioite and trithioate defoliants did not differ from each other because the trithioite, relatively unstable, is converted to trithioate in the presence of oxygen (9). Therefore *S, S, S*-tributylphosphorotrithioate, commercially sold as DEF, was used throughout the study and was synthesized with either the P^{32} or S^{35} label as described previously (12).

Physiological Studies: Ten pots, each containing two mature cotton plants (Deltapine variety) with approximately one-third of their bolls opened, were divided into two equal groups. These plants were used to determine the effect of DEF on leaf respiration and on the sulfhydryl groups of the water soluble proteins in the leaf. One group of plants served as a control while the other received unlabeled DEF at the equivalent rate per acre of 3 pounds of DEF emulsified in 35 gallons of water.

Lots of ten disks, selected at random from the treated and control leaves, were cut with a No. 6 cork borer at 4, 12, 24, 48, and 72 hours after treatment. Duplicate Warburg flasks, with five disks each of control and treated leaves, were prepared to determine the total oxygen uptake for each time period by the direct method of Warburg as described by Umbreit (13). Respiration was measured every half hour for 2 hours at 25 C. After completing the experiment, the dry weights of the tissues were determined after a constant weight had been attained in a force draft oven at 80 C.

A comparison of the water soluble sulfhydryl content of untreated, young, mature, and senescent cotton leaves was made with leaves of the same age group treated with DEF. Randomized 5 g samples of young leaves from the top section of the plant, mature leaves from the mid section, and yellow abscising leaves (from the base of the control plants only) were harvested from both groups of plants at 0, 24, 48, and 72 hours after treatment. Each fresh sample was macerated with 20 ml of water at 10 C in a Waring blendor for 5 minutes. After the broken cellular material was centrifuged ($822 \times g$ for 5 min), the clear supernatant was made up to exactly 25 ml. Aliquots of the latter (½ ml) were used for the sulfhydryl determination by the manometric method described in detail by Ishii (8). The amount of water soluble protein for each extract was determined by the method of Loomis and Schull (11).

Extraction; Chromatographic Studies: Two groups of plants, similar to those employed in the physiological studies, were used to determine the mode and rate of DEF degradation. One group was sprayed with the P^{32} labeled compound (0.37 mc/mmole) and the other with the S^{35} labeled compound (0.22 mc/mmole) at the equivalent rate per acre of 3 pounds of DEF emulsified in 35 gallons of water. Randomized leaf samples of 5 g were then harvested from each group at 4, 12, 24, 48, and 72 hours after treatment. These samples were frozen, lyophilized, and ground to pass a 60 mesh screen. Abscised leaves, most of which were nearly dry, were collected a week after treatment and ground to 60 mesh fineness without the preliminary lyophilization procedure. The greenhouse temperature ranged from 25 to 27 C during the entire experiment.

[1] Received May 22, 1961.

In order to follow quantitatively the rate of DEF degradation in the leaves with time, the lyophilized leaf samples were extracted with polar and non-polar solvents and the extracts in turn were assayed for total P^{32} or S^{35} activity. Before extraction, however, the total P^{32} or S^{35} activity in terms of counts per minute per gram of lyophilized leaf material was determined by use of a gas flow proportional counter along with application of the usual quantitative techniques (1). Duplicate amounts (0.5 g each) from each lyophilized sample were then extracted for 5 minutes in an Elvejhem-Potter glass homogenizer with 5 ml of water. Additional duplicates of the same weight from the lyophilized samples were extracted with 5 ml of chloroform in a similar manner. After centrifuging both the water and chloroform extracts (822 × *g* for 5 min) and adjusting the supernatants to 10 ml, suitable aliquots were evaporated for counting. The usual quantitative techniques (1) were observed to determine the total activity of P^{32} or S^{35} in the extracts.

To determine the relative distribution of radioactivity among the S^{35} and P^{32} labeled breakdown products of DEF in the leaves during the time of treatment, the extracts from above as well as additional water, and chloroform extracts from the week old abscised leaves were separately concentrated and subjected to ascending paper chromatography. The water extracts were deproteinized by adding an equal amount of acetone (25 C) and were concentrated under vacuum to 2 ml at 25 C. (Preliminary experiments indicated that no significant amount of radioactivity was adsorbed on the protein precipitates). Portions (20 μl) of this concentrate were applied to strips of Whatman No. 3 filter paper (28 × 2 cm) until a cpm value of 1,000 was reached. The strips were then developed in a solvent system composed of *n*-butanol, acetic acid, and water (100:22:50 V/V). The chloroform extracts were concentrated to 2 ml in an evaporating dish at 25 C. Ten microliter amounts of this concentrate were applied (until 1,000 cpm) to the same type of filter paper strips and developed by the solvent systems of Fukuto et al. (4). The developed strips for both types of extracts were cut into 1 cm lengths and counted in a gas flow proportional counter. Plots were made of the counts per minute versus Rf values.

Results & Discussion

Physiological Studies: The effect of DEF upon the respiration of leaf blade tissue is shown in figure 1. The straight heavy line represents the average oxygen uptake of the control leaves during the given time period. The dotted lines which flank both sides of the heavy line represent the standard error of the mean arising from experimental error and from the natural changes in the respiration rate. The total oxygen uptake of DEF-treated leaves starts to increase significantly and reaches a maximum value 24 to 48 hours after treatment. With the onset of leaf separation (48–72 hours), respiration begins to drop toward the control mean. These results agree with previous work on chemical defoliants and their effect on respiration (10).

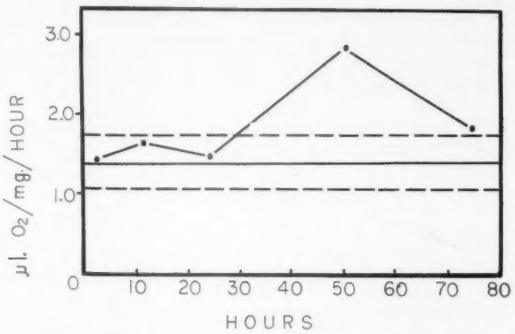

Fig. 1. The average level of oxygen uptake in untreated cotton leaves as compared to that of leaves treated with DEF.

The solid straight line flanked by the dotted boundaries in figure 2 represent the average levels, with their standard error, of micrograms of -SH per milligram of water soluble protein in leaves of various ages. Apparently as the leaf ages, its specific amount of -SH groups diminishes. Note in figure 2 that just before and during induced abscission (48–72 hr) the specific -SH content in both young and mature leaves treated with DEF drops to or below the specific level of -SH contained in the natural senescent and partially abscising basal leaves.

Although the evidence is indirect, there appears to be a correlation of abscission to the specific level of

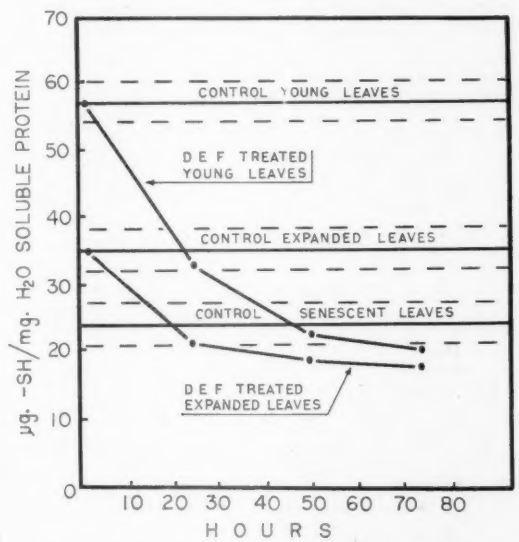

Fig. 2. Average micrograms of -SH per mg. of water soluble protein in untreated young, fully expanded, and senescent cotton leaves as compared to -SH specificity in DEF treated leaves sampled at the time intervals indicated.

TABLE I

RELATIVE DISTRIBUTION OF RADIOACTIVITY OF FRACTIONS OBTAINED FROM PAPER CHROMATOGRAMS OF P^{32} & S^{35} LABELED WATER EXTRACTS *

Hr AFTER APPLICATION	P^{32} FRACTIONS			S^{35} FRACTIONS		
	0.58**	0.70**	0.83**	0.57**	0.65**	0.84**
4	167	65	830	634	266	65
24	500	390	672	630	902	233
48	903	534	365	834	467	264
165	668	165	300	932	235	101

* Results, expressed as counts per minute, represent an average of two separate experiments for both the P^{32} and S^{35} labeled DEF.
** Rf values of the radioactive fractions.

water soluble -SH groups in the leaf tissue. This same observation has been noted for ethylene, another potent defoliant (7).

METABOLITE FORMATION & DEGRADATION RATE OF DEF: When plots of the chromatographed chloroform extracts were compared to those obtained from standard samples of S^{35} and P^{32} labeled DEF chromatographed in the same solvent systems, DEF was the only compound in the chloroform extracts. Other workers (2) have shown that chloroform soluble metabolites from organophosphorus compounds similar in structure to DEF were separated from one another and from their parent compounds by the chromatographic systems employed in this study. Since no metabolites of this nature were found in our chloroform extracts, it was apparent that DEF was directly degraded into water soluble products.

Relative distribution of radioactivity (table I) of the fractions on paper chromatograms from the water extracts show a definite sequence of formation with time for both the P^{32} and S^{35} labeled degradation products of DEF. Based upon similar Rf values, it appears that some of these water soluble products might contain both the S^{35} and P^{32} label. A micro elemental analysis for phosphorus (3) on all three S^{35} labeled fractions however, failed to reveal the presence of this element. Fractions with Rf values of 0.58 and 0.57 from the P^{32} and S^{35} labeled DEF, respectively, were the only compounds of appreciable quantity in the week old abscised leaves. Apparently these metabolites were not as readily translocated from the leaf as were the other degradation products.

Thus, it appears that before leaf abscission takes place, DEF is directly converted into three water soluble phosphorus and three water soluble sulfur degradation products. We found that mild in vitro acid hydrolysis (0.1 N HCl at 100 C under reflux for 4, 12 & 24 hr) yielded only two products, namely phosphoric acid and sulfur containing hydrocarbon. Apparently these hydrolysis products, if formed in vivo, are combined with certain plant metabolites to give the additional degradation products noted. Whether any one or all of the degradation products are directly or indirectly responsible for initiating leaf abscission is not known.

If one plots the log of the counts per minute of S^{35} or P^{32} versus time, the total amounts of P^{32} or S^{35} in both the leaf powder and chloroform extracts from table II decrease with time according to the integrated form of the well known differential equation, $dx/dt = kx$ (x = amount of P^{32} or S^{35} present at any given time t; k = rate of decrease).

Inasmuch as DEF is converted directly into water soluble products, the k value for the rate of decrease of the chloroform soluble P^{32} or S^{35} labeled compounds was used to estimate the specific rate of DEF degradation. The k values for both P^{32} and S^{35} com-

TABLE II

QUANTITATIVE EXTRACTION OF P^{32} & S^{35} FROM COTTON LEAVES & FRACTIONATION INTO POLAR & NON-POLAR SOLVENT SOLUBLE CONSTITUENTS*

Hr AFTER APPLICATION	TOTAL ISOTOPE IN LEAF POWDER		CHLOROFORM SOLUBLE ISOTOPE		WATER SOLUBLE ISOTOPE	
	P^{32}	S^{35}	P^{32}	S^{35}	P^{32}	S^{35}
0	873,000**	630,000**	710,000**	507,000**	...	...
4	890,680	...	695,000	...	9,680	...
12	...	600,200	...	445,900	...	19,550
24	643,830	506,300	500,000	406,000	18,050	21,280
48	617,530	405,400	399,000	234,800	35,100	20,300
72	...	372,000	...	232,900	...	22,250

* Results, expressed as counts per minute of isotope per gram of leaf powder, represent an average of two separate experiments for both the P^{32} and S^{35} labeled DEF.
** Extrapolated values from least squares plot of counts per minute per gram versus time in hours.

pounds were found from the slopes of straight lines which resulted from the plots of the chloroform extracts as mentioned above. The regression equations of these lines for both P^{32} and S^{35} were calculated by the method of least squares and these equations along with their k values in reciprocal hours are as follows: For the P^{32} labeled chloroform extract, $Y = 5.851 - 0.00542\ X$ ($k = -0.0125$) and for the S^{35} labeled chloroform extract, $Y = 5.705 - 0.00530\ X$ ($k = -0.0122$). Loss of the parent compound due to volatilization and translocation was small and was not corrected for in calculating the k values.

The average value of k from the above values for the degradation of DEF is 0.0124. This means that approximately 1.2 % of the total DEF in the leaves at 25 to 27 C will decompose to water soluble products in 1 hour or in terms of $T_{½} = 0.693/k$, DEF has a half life of about 56 hours. Approximately the same values of k were found when a rate as low as one or as high as 4 pounds of DEF per acre was used.

Summary

I. There was a significant stimulation of respiration of DEF treated leaves before abscission took place.

II. DEF reduced the specific sulfhydryl content of young and mature cotton leaves to that level found in senescent and naturally abscising cotton leaves.

III. We found three water soluble sulfur and three water soluble phosphorus degradation products of *S, S, S*-tributyltriphosphotrithioate (DEF) in cotton leaves, but no chloroform soluble metabolites.

IV. The approximate degradation rate for DEF was 1.2 % of the total amount in 1 hour.

Acknowledgments

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CARBOHYDRATE TRANSLOCATION IN RASPBERRY & SOYBEAN [1, 2, 3]

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INTRODUCTION

In recent years, renewed interest has been shown in the area of translocation of organic compounds in the phloem, both from the standpoint of mechanism and species of molecules transported. Several reviews concerning the subject have appeared including the critical discussions of Arisz (1) and Crafts (3), the anatomical review of Esau et al. (6), and the comprehensive treatment of the subject by Swanson (10). In addition, Zimmermann (15, 17, 18) has reviewed the special aspect of carbohydrate transport in trees. One of the hypotheses proposed in these and other discussions, suggests that sucrose and sucrose-containing oligosaccharides are the major transport compounds in most higher plants. In addition, it has been proposed that the hexoses found in bark tissue result primarily from interconversions of the transport molecules. During the 1930's Mason and Maskell (8), in their work with cotton, and Engard (5), studying raspberry, inferred that sucrose was the major transport compound in the phloem together with secondary amounts of hexose. This conclusion was based on quantitative measurement of the ratio of reducing to non-reducing sugars in bark tissues. Unfortunately, such measurements may bear little or no relation to circumstances existing in the transport stream. Quite recently two somewhat more elegant methods have been developed to distinguish between molecules in transit and those in ambient pools. One of these is based on the premise that the so-called sieve-tube exudate is a valid sample of the translocation stream. Early exudate analysts such as Moose (9) report the presence of reducing sugars, but these studies are of questionable validity owing to the methods used for carbohydrate identification and the absence of sufficient precautions to prevent chemical changes. Recent applications of this technique, such as those of Kennedy and Mittler (7), Wanner (13), Ziegler (14), and Zimmermann (16), are much more reliable. Zimmermann (18) has divided exudates into three groups. The first group contains only sucrose. The second group contains oligosaccharides of the raffinose family and lesser amounts of sucrose. The third group's exudate primarily contains sucrose with small amounts of raffinose family oligosaccharides. Exudate from more than 250 species have been analyzed and significantly, glucose and fructose have been found in only a few instances and then in minute quantities.

Another approach has been the use of radioactive isotopes. Owing to their unique properties, radiotracers are particularly well suited to the problem under discussion, since labeled compounds can be distinguished as recent additions to the tissues in which they are detected. Several pertinent investigations have been made with this technique. Vernon and Aronoff (2, 12) studied translocation of carbon-14-labeled photosynthate in soybean phloem. They concluded that sucrose was the major translocated carbohydrate and that glucose and fructose were translocated to a lesser degree.

Although the above study casts some doubt on the sucrose-oligosaccharide transport theory, the subsequent work of Swanson and El Shishiny (11) appears to support the hypothesis. They postulated that C^{14}-labeled glucose and fructose found in the bark were produced by hydrolysis of the translocated sucrose and consequently the ratio of C^{14}-glucose to C^{14}-fructose should be unity. Further, the ratio of C^{14}-hexose to C^{14}-sucrose should decrease in the phloem with distance from the supply. These proposed relationships were shown to exist in grape (11).

The two experiments which follow extend the approach of Swanson and El Shishiny to other species. Raspberry was chosen because Engard (5) reported very high hexose to sucrose ratios in this species. Soybean was investigated as a result of the apparent lack of unity of C^{14}-fructose: C^{14}-glucose ratios reported by Vernon and Aronoff (12).

METHODS & MATERIALS

In March 1956, 8 two-year-old nursery plants of Canby variety raspberry (Lloyd George x Viking) were planted in 12-inch pots. The potting mixture consisted of one part loam, one part peat moss, and three parts sand. The plants were placed in the greenhouse and watered daily. An application of a commercial fertilizer known as Ra-Pid-Gro was made every 2 weeks. At the end of June, when the canes averaged 4 feet in length, one 126 cm long cane was selected for the experiment. The C^{14} supply leaf was located approximately 55 cm from the tip.

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[2] This work was supported in part by a grant from the Rockefeller Foundation to Dr. C. A. Swanson through the Ohio State University Research Foundation. A part of the research reported here appears in the doctoral dissertation of the author.

[3] Paper from the department of Botany and Plant Pathology, The Ohio State University, No. 649.

The soybean plants (*Glycine max* (L.) var. Hawkeye) grew in a controlled environment room. The seeds were germinated in moist vermiculite and the seedlings subsequently transferred to black-painted, aluminum foil-wrapped Mason Jars containing aerated nutrient solution of the following composition: 1×10^{-3} mole KH_2PO_4; 5×10^{-3} mole $Ca(NO_3)_2$; 2×10^{-3} mole $MgSO_4$; 5×10^{-3} mole KNO_3; 4.6×10^{-5} mole H_3BO_3; (0.5 ppm B); 9.2×10^{-6} mole $MnCl_2$; (0.5 ppm Mn); 8.1×10^{-7} mole $ZnSO_4$; (0.05 ppm Zn); 2.9×10^{-7} mole $CuSO_4 \cdot 5H_2O$; (0.018 ppm Cu); 5.7×10^{-7} mole $NaMoO_4$; (0.055 ppm Mo), and 1×10^{-4} mole EDTA-Fe (5 ppm Fe).

The controlled environment room was operated on a photoperiod of 14 hours of light and 10 hours of darkness. The temperature was maintained at 25 C during the light period and 19 C during the dark period. Light was supplied by 36 cool white, 96T8 fluorescent tubes and 10 sixty-watt tungsten bulbs. This produced approximately 1,000 ft-c of illuminance, measured 7 inches above the table.

The experimental plant was selected 35 days after the seed was sown and had four fully expanded trifoliate leaves. It measured 22 cm from cotoyledonary nodes to the tip. The C^{14} was supplied to the most recently expanded trifoliate leaf.

In each of the experiments C^{14}-labeled CO_2 was generated in a closed system patterned after that of Swanson and El Shishiny (11). In this arrangement, carbon dioxide can be released, circulated through the leaf chamber, and any excess absorbed in sodium hydroxide, without opening the system.

The total amount of radioactive carbon used varied with the experiment. A total of 1.3 millicuries of C^{14} was used for the raspberry experiment and 1.8 millicuries for the soybean. Sufficient carrier $BaCO_3$ was added to the radioactive material to produce an initial carbon dioxide concentration of 1.5 % in the atmosphere circulating through the system. This totaled 172 ml of carbon dioxide.

Carbon 14 exposure was terminated at the end of 1 hour in the raspberry experiment and after 35 minutes in the soybean experiment. In each experiment, activity was detectable at this time, 10 cm above and below the supply leaf using a 1.5 mg/cm² geiger tube attached to a Nuclear-Chicago Model 2612 rate meter.

At the end of the exposure period, each plant was sectioned, the bark separated from the xylem, and treated essentially as described by Swanson and El Shishiny. To insure minimal biochemical changes in the material, the bark and xylem samples were frozen in liquid nitrogen within 10 minutes after removal from the plant. The tissues were subsequently lyophilized and stored in glass ampoules until the time of analysis.

Preparatory to analysis, a sample was ground in a micro-mortar and, when possible, the entire sample used. An aliquot was taken if the size of the sample exceeded the 80 mg capacity of the micro-extraction apparatus. Ethanol, 80 %, was used to extract the sugars from the tissue; 125 μg of rhamnose in 80 % ethanol were added as an internal check on losses occurring during analysis. Rhamnose does not occur as a free sugar in either of the test plants and has an Rf quite different from that of sucrose, glucose, and fructose. The extract was transferred quantitatively to a 5-ml beaker, dried under a stream of nitrogen, vacuum desiccated, and then extracted with anhydrous ether. The residue was then dissolved in 250 μl of distilled water and the resulting solution spotted on Whatman No. 1 chromatography paper. Two chromatograms were prepared for each sample (100 μl aliquots on each). The chromatograms were developed for 72 hours by the continuous descending method, using *n*-butanol-ethanol-water (45:5:50 parts by volume). Guide strips on both edges of the chromatograms were spotted with 15 μl of solution containing 150 μg each of sucrose, glucose, fructose, and rhamnose. After developing the chromatograms, the guide strips were cut off and sprayed with a solution of acetic acid, trichloracetic acid, and benzidine and then placed in an oven at 100 C for 5 minutes. The resulting spots were used to cut appropriate strips from the main body of the chromatograms. Each strip was eluted with approximately 0.5 ml of distilled water. The resulting solutions were adjusted to a total volume of 1 ml with distilled water and 100 μl aliquots plated for counting. Chemical analyses of the remaining fractions were made by the method of Dimler et al. (4).

Since the raffinose-family oligosaccharides remained near the origin of the chromatograms under the conditions described above, several samples from each experiment were chromatographed using butanol, acetic acid, and water (3:3:2). This solvent system separates the raffinose-family oligosaccharides from each other and from sucrose. Half of each chromatogram was spotted with known raffinose. Since a commercial supply of stachyose was not available, an 80 % ethanol extract of soybean seeds was spotted on the same half of the chromatogram with the raffinose. Soybean seeds contain relatively large amounts of stachyose.

Counting was done with a Nuclear-Chicago Model 161A Scaler equipped with a 1.5 mg/cm² end window geiger tube shielded with a Model 3035 sample holder.

TABLE I

CONCENTRATIONS OF SUGARS (LABELED & UNLABELED) IN BARK OF RASPBERRY RELATIVE TO ACROPETAL TRANSLOCATION DISTANCE FROM CARBON 14 SUPPLY LEAF

DISTANCE OF TRANSLOCATION*	SUGARS AS % SAMPLE DRY WT		
	SUCROSE	GLUCOSE	FRUCTOSE
66 mm	2.09	0.74	0.56
152 mm	2.91	0.93	0.88
224 mm	2.41	0.81	0.65
300 mm	2.28	0.69	0.61
365 mm	1.64	0.51	0.44
480 mm	1.05	0.24	0.19

* Distance from supply leaf node to center of sample.

TABLE II

RELATIVE CONCENTRATIONS & SUCROSE TO HEXOSE RATIOS OF CARBON 14-LABELED SUGARS IN BARK OF RASPBERRY AS FUNCTIONS OF TRANSLOCATION DISTANCES

DISTANCE OF TRANSLOCATION	cpm/mg DRY WT OF BARK				
	SUCROSE	GLUCOSE	FRUCTOSE	GLU/SUC	FRU/SUC
66 mm	5400	950	930	0.176	0.172
152 mm	2995	320	331	0.107	0.110
224 mm	2097	190	194	0.091	0.092
300 mm	1720	100	93	0.058	0.054
365 mm	1320	67*	70*	0.050	0.053
480 mm	750	33*	35*	0.044	0.046

* The amount of activity in these samples was too low to count to plus or minus 1 % at the 95 % confidence level.

Sufficient counts were totalized, when possible, to insure accuracy of ± 1 % at the 95 % confidence level.

Several chromatograms were placed against Eastman Kodak No-Screen X-ray film in standard X-ray film casettes for radioautography. These radioautographs were used to test the accuracy of the guide strip method for locating compounds in the center of the chromatogram and as a more sensitive assay for small amounts of oligosaccharides which might not be detected with spray reagents.

TABLE III

CONCENTRATIONS OF SUGARS (LABELED & UNLABELED) IN BARK OF SOYBEAN RELATIVE TO TRANSLOCATION DISTANCE FROM CARBON 14 SUPPLY LEAF

DISTANCE OF TRANSLOCATION*	SUGARS AS % SAMPLE DRY WT		
	SUCROSE	GLUCOSE	FRUCTOSE
13 mm Above supply	0.96	1.11	1.12
35 mm Above supply	0.80	0.40	0.41
15 mm Below supply	0.93	1.31	1.33
45 mm Below supply	1.28	0.146	0.29
83 mm Below supply	1.30	0.26	0.23

* Distances are from the C^{14} supply leaf node to the center of the sample.

RESULTS & DISCUSSION

In tables I and III are the data obtained from colorimetric analyses of the total amounts of sucrose, glucose, and fructose in the bark of raspberry and soybean. Unlike the data of Engard (5), the hexose to sucrose ratios are of the same order of magnitude as those observed in other species such as soybean and grape. Although this experiment has been repeated with Cuthbert variety raspberry, the same results were obtained. The more likely reasons for this disagreement with Engard's data are the greater precautions against chemical changes and possible variations in sugar ratios at different stages of development.

The radio-chemical data (tables II & IV) are in excellent agreement with those obtained by Swanson and El Shishiny from grape.

The straight line function between distance of translocation and decrease of radioactivity is graphically represented in figures 1 and 2. The increasing ratio between radio-sucrose and hexose predicted by the hypothesis of Swanson and El Shishiny, is reflected in the divergence of the sucrose and hexose curves. If this divergence is actually a reflection of a slower translocation rate of the hexoses rather than the result of sucrose inversion, then the glucose and fructose apparently are moving at equal rates.

An explanation for the lack of agreement between the soybean data reported here and that of Vernon and Aronoff (12) is not readily available. Examination of the published radioautograph of one of their chromatograms suggests the possibility that there was some overlapping of sucrose and glucose spots. For this reason it may have been difficult to accurately assess the radioactive glucose fraction.

Analyses of raspberry and soybean for the presence of raffinose family oligosaccharides were made. Both species contained raffinose in stem and leaf tissues. The amount detected was so small that spray reagents did not react in sufficient quantity to be observed. Identification was, therefore, made by radioautography. A second radioactive band slightly above raffinose was also found. However, this second band did not coincide with the so-called soybean seed stachyose spot on the chromatogram. Until a pure source of stachyose is obtained, identification is uncertain; it appears that stachyose is not present in the leaves or stems of either raspberry or soybean.

TABLE IV

RELATIVE CONCENTRATIONS & SUCROSE TO HEXOSE RATIOS OF CARBON 14-LABELED SUGARS IN BARK OF HAWKEYE SOYBEAN AS FUNCTIONS OF TRANSLOCATION DISTANCES

DISTANCE OF TRANSLOCATION	cpm/mgm DRY WT OF BARK				
	SUCROSE	GLUCOSE	FRUCTOSE	GLU/SUC	FRU/SUC
13 mm Above supply	4,679	372	377	0.079	0.080
35 mm Above supply	2,323	130	135	0.055	0.058
15 mm Below supply	1,774	880	890	0.496	0.501
45 mm Below supply	1,250	341	338	0.272	0.270
83 mm Below supply	510	124	131	0.243	0.257

If this observation is correct, then large amounts of stachyose must be synthesized within the soybean seed from other transport molecules or storage forms of carbohydrate.

On the basis of the data reported here, both raspberry and soybean belong in Zimmermann's Group III in which sucrose together with small amounts of raffinose are the transport molecules of the phloem. Attempts are being made to confirm this classification with sieve-tube exudate analyses.

In conclusion, it is strongly suggested from evidence from the present study that sucrose is the major transport sugar in raspberry and soybean. In addition, small amounts of raffinose probably are translocated in the phloem. The radio-glucose to radio-fructose ratios imply that these sugars are derived primarily from hydrolysis of sucrose.

Summary

To test the hypothesis that sucrose is the primary form in which carbohydrate is translocated in soybean and raspberry, experiments were performed using the approach of Swanson and El Shishiny (11). Glucose and fructose were radio-chemically equal; the ratio of sucrose to hexoses increased with translocation distance. These relationships support the hypothesis that sucrose is translocated and that the glucose and fructose found in the phloem result from hydrolysis of sucrose.

Small amounts of the oligosaccharide raffinose also were found in both raspberry and soybean. A radioactive substance with an Rf slightly smaller than that of raffinose also was detected. This band, unidentified, probably is not a member of the raffinose family.

Acknowledgments

The author expresses his appreciation to Dr. Kenneth Webb, Dr. C. A. Swanson, and Dr. B. S. Meyer for their criticisms and discussions concerning this research and the preparation of the manuscript.

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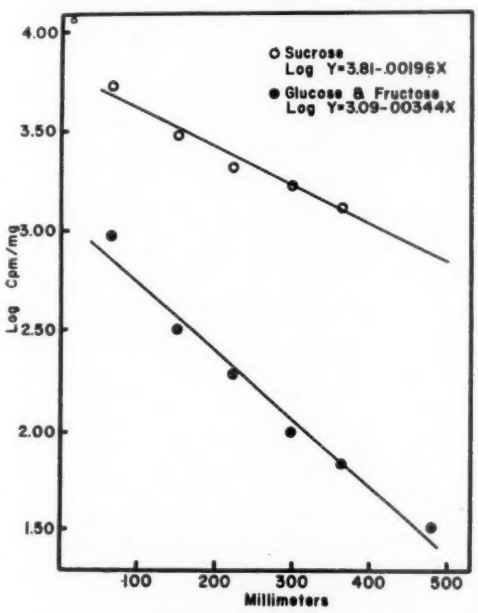

Fig. 1. Least squares fit regression lines of log activity as a function of translocation distance. Raspberry.

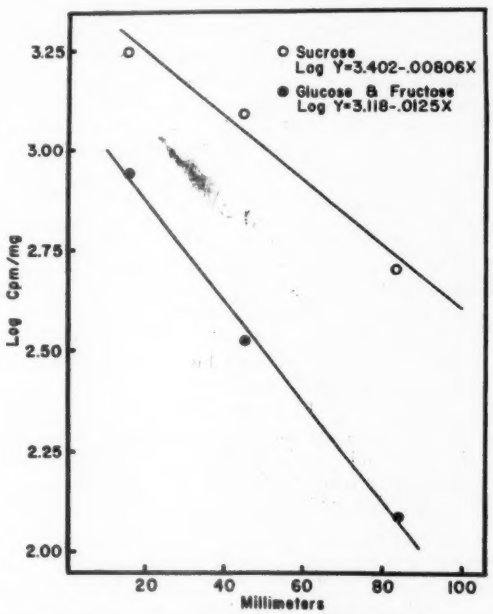

Fig. 2. Least squares fit regression lines of log activity as a function of translocation distance. Soybean.

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WESTERN X-LITTLE CHERRY VIRUS EFFECTS ON RESPIRATION OF HOST TREE[1, 2]

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A virus disease common to several Prunus species has been described by Blodgett (2), Zeller and Evans (12), Reeves and Hutchins (8), and others (7) on peach, sweet and sour cherries, chokecherries, and ornamental cherries. The disease has been named Western X-disease although it goes by other names in local areas. In peach the disease produces a severe decline which ends in the death of the tree. In sour cherries the disease results in a decline which may or may not end in the death of the tree, depending largely on the rootstock to which it has been grafted, and in sweet cherries the disease may or may not produce a noticeable decline. However, a common factor in all of the cherries is the failure of the fruit to develop to ripeness. A small fruit of a characteristic pointed shape, poor or no color, and containing very little carbohydrate is produced. Such fruits appear to develop normally until about two weeks before maturity and then apparently cease any further development and remain small and relatively colorless. They may persist on the tree for several weeks in this condition, without apparent change, long after normal fruits have dried and dropped from the tree. Several theories have been advanced to account for this phenomenon; the most commonly cited is the study of Schneider (9) who attributed these effects to phloem breakdown with resultant reduction in translocation.

Once a tree branch has been infected, the virus persists indefinitely, but does not appear to affect every cherry. Not infrequently a single cherry out of a cluster will appear to develop normally and the rest remain small and colorless. Only occasionally will a branch be found that will have no normal-appearing fruit.

Phloem blockage has been the most usual explanation for the virus effects observed in this disease. Nevertheless, studies in this laboratory showed no evident abnormality of the phloem tissue and preliminary studies with C^{14} showed only small impairment of translocation. Since failure of translocation seemed an inadequate explanation for the effects observed, a study was undertaken to determine whether the virus was producing either a direct or an indirect effect on the respiratory mechanism.

Methods & Materials

All the respiratory rate studies were done at The Dalles in Oregon, an area in which the disease is

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[2] Technical Paper No. 1230, Oregon Agricultural Experiment Station.

common, in order to have a constant supply of fresh materials in the immediate vicinity of the laboratory. The samples were taken from trees on which virus infection records had been kept for several years. Samples of diseased material, leaves, stems, petioles, pedicels, fruit pericarp, and embryos were removed from branches showing severe symptoms of infection and showing severe symptoms for at least a year previously. Control samples of healthy tissues were taken from trees showing no symptoms of disease and, to avoid the possibility of a symptomless infection, surveyed during at the least the succeeding 2 years and found free of virus symptoms.

A number of sampling methods were tried in the preliminary stages of this investigation. Random sampling was difficult since active disease symptoms might be limited to a particular limb or section of a tree. Consequently, the samples were made as randomly as possible within that part of the tree known to be infected. Only samples of diseased fruit showing typical symptoms from trees of known infection were used. Usually ten fruits were included in any particular sample; all samples were paired, as for instance the fifth leaf from a healthy branch against a fifth leaf from a diseased branch. Samples, collected at times when there were no fruits on the tree, were taken from spurs marked during the previous fruiting period. Samples were taken from trees of comparable size and vigor growing in the same habitat, usually not separated from each other by more than 125 feet.

Since preliminary studies had indicated that the respiratory rate of leaves was effected to a small extent by the age and position of the leaf, all comparisons of these tissues were based on leaves of the same age and at the same relative position on the spur or branch. Leaf tissue and woody tissue were chosen especially carefully.

Leaves were sampled either as half leaves or leaf punches. Opposite leaf halves were used for duplicate samples and opposite punchings were similarly chosen. Woody tissue samples were taken from 1st year actively growing branches from trees of comparable vigor. Fruit tissue was sampled by removing 2 mm thick slices and washing them for about ten minutes in several changes of well water. Well water did less damage than distilled water and gave more consistent results than 10 % or 20 % sucrose solution. Embryo tissue was sampled by removing the fleshy part of the fruit and cracking the hard pit with a special cracking pliers modified to limit the closure of the jaws to a predetermined value. Any kernel showing damage was discarded even though this was shown to be an insignificant source of error.

The rate of respiration was studied by the direct method of Warburg (10). The temperature of the waterbath was 30 C. Usually tissues such as leaves, petioles, and spurs were not suspended in liquid medium; soft tissues were suspended in buffered solutions. In order to use inhibitors or substrates, two methods of application were used. In the first method the tissue was immersed to half its depth in a solution of the inhibitor in a petri dish and was allowed to stand for 16 hours. In the second method, the tissue was immersed in a test tube containing the inhibitor solution; a vacuum was applied for five minutes after boiling appeared in the tubes; the vacuum was released, and the tissue was allowed to remain in the inhibitor solution for an additional 10 minutes. The tissue was then removed, drained on a paper towel, and weighed. In general, the second treatment was more suited to these experiments since there was less delay in handling the materials. With either method there is some difficulty in getting a known amount of the inhibitor into the tissues. A large number of experiments at various concentrations of inhibitor indicated that the method gave sufficiently reproducible results to compare diseased and healthy tissues.

Fruit tissue was suspended in either 10 % sucrose solution or M/100 phosphate buffer pH 3.5; the latter gave more consistent results. Other tissues were not suspended in any solution since normally they are exposed to air and a liquid might interfere with oxygen diffusion. One milliliter of water in the sidearm of the Warburg vessel kept the atmosphere saturated. All the experiments were run in air since 100 % oxygen did not enhance the rate of oxygen uptake and even proved somewhat inhibitory after several hours. Where possible, the samples were saved for analysis of acid soluble inorganic phosphate and total phosphate. In early experiments the method of Fiske and Subbarow (4) was used, but later the method of Pons et al. (6) was adopted. In either method the blue color was read on a Model DU Beckman spectrophotometer at a wavelength of 730 mμ. To determine total soluble phosphate, aliquots of the trichloroacetic acid extract were dried in microKjeldahl flasks, ashed with H_2SO_4 and 30 % H_2O_2, and analyzed as above.

Extraction of the acid soluble phosphate presented some difficulty. Samples were ground in a mortar with a known quantity of ice cold 0.75 N trichloroacetic acid; the resulting mixture was stirred in an ice bath for 15 minutes. The sample was then centrifuged and turbidity was removed by filtering through a 1 mm layer of so-called filter aid in a fine porosity pyrex sintered funnel.

Results

The respiratory rate of vegetative tissues from trees infected with virus differed little from that of comparable tissues from healthy trees. To determine whether or not carbohydrate might be limiting because of a failure of translocation, as suggested by Schneider (9), tissue samples were immersed in or vacuum infiltrated with 0.5, 1.0, 210, 5.0, and 10 % sucrose and 0.01 to 0.001M KH_2PO_4, either alone or in combination. No differences were discovered with these treatments. In general, all of these treatments caused about a 10 % depression in respiratory rate.

TABLE I

RATE OF RESPIRATION OF VEGETATIVE TISSUES FROM HEALTHY TREES & TREES INFECTED WITH WESTERN X-LITTLE CHERRY VIRUS.

VARIETY	TISSUE	μl O_2/g FR TISSUE/HR RESPIR. HEALTHY	DISEASED
Montmorency	Leaves	651	726
Bing	Leaves	734	699
Montmorency	Petioles	153	156
Montmorency	Pedicels	249	250
Montmorency	Fruit slices 2 mm	92	117
Royal Anne	Fruit slices	88	109
Montmorency	Fruit spurs	366	375
Montmorency	Wood (young)	182	177
Montmorency	Wood (old)	82	79

The virus evidently causes relatively little disorganization of the respiratory mechanism of vegetative tissues. In table I are the relative rates of oxygen uptake for a number of healthy and infected tissues. These results represent averages of a number of duplicate determinations of paired samples. The slightly greater respiratory rate of the diseased Montmorency leaves in both recent and old infections probably represents the greatest effect of virus found on any vegetative tissue and is probably related to the smaller cell size of the infected leaves. The slightly lower rate of respiration for infected leaves from Bing trees probably represents a valid difference, since these results were obtained from carefully paired samples from three trees on which Bing limbs had been grafted high on Mahaleb rootstocks which do not transmit the virus. Those healthy samples which grew on the same rootstock presumably had the same nutritional status as the diseased.

In the early stages of fruit development, no differences in external or internal appearance between healthy and diseased fruits were detected. The first differences were observed when the fruit began to accumulate carbohydrate and expand in size prior to ripening. During this period there was a rapid accumulation of sugar in the fleshy part of the healthy fruit and a similar accumulation of fat in the embryo. The virus infected fruit accumulated little or no sugar and remained small and colorless. It is from this behavior that the disease gets its name.

Respiratory rates of the fleshy part of diseased and healthy fruit were difficult to compare. Although differences were found, the physical and chemical states of the tissues were not sufficiently alike to make a comparison possible. The large, thin-walled, succulent cells of the healthy fruit were difficult to compare with the small, firm cells of the diseased tissue either on a fresh weight, dry weight, or nitrogen content basis. Considerable amounts of asparagine in the diseased tissue made the last method particularly unsuitable.

Diseased embryos did not accumulate fat as did the embryos in apparently healthy fruits. A considerably lower respiratory rate was also found in the diseased embryo. In most cases the respiratory rate of these embryos was less than half that of the control, and in many cases a third or even less (fig 1). Since at least part of the energy of respiration of these plant parts is employed in accumulation and synthesis within the fruit, developmental failure might well be correlated with this lower rate of respiration.

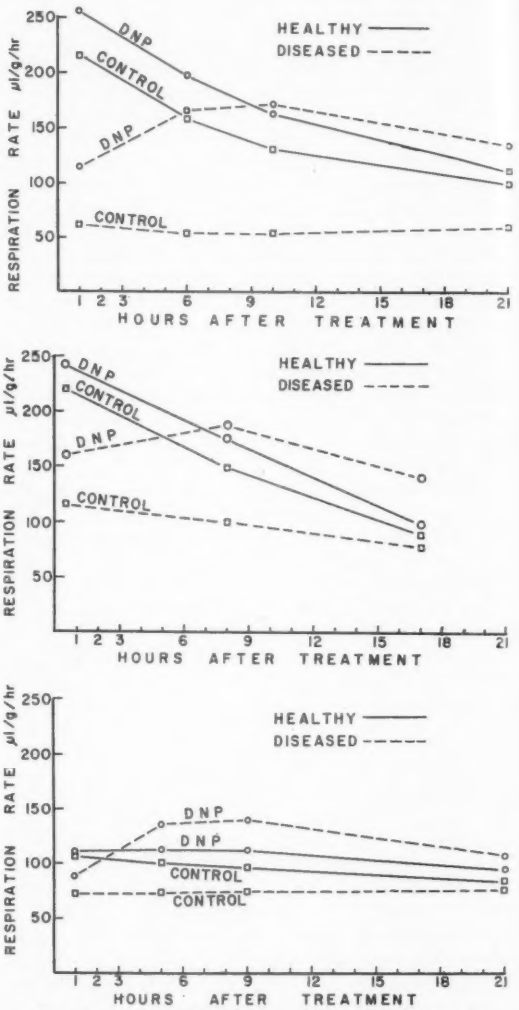

FIG. 1a (*top*). Respiratory rate in CO_2-free air at 30 C of immature cherry embryos with and without treatment with 0.001 M DNP.

FIG. 1b (*center*). Respiratory rate in CO_2-free air at 30 C of nearly mature cherry embryos with and without treatment with 0.001 M DNP.

FIG. 1c (*bottom*). Respiratory rate in CO_2-free air at 30 C of mature cherry embryos with and without treatment with 0.001 M DNP.

TABLE II

RESPIRATORY QUOTIENT OF EMBRYOS FROM HEALTHY & DISEASED CHERRY FRUITS INFECTED WITH WESTERN X-LITTLE CHERRY.

VARIETY	RESPIRATORY QUOTIENT	CONDITION
Royal Anne	1.54	Healthy
	0.82	Diseased
Lambert (nearly mature)	1.12	Healthy
	0.79	Diseased
	1.04	Healthy treated with 0.001 M DNP
	0.94	Diseased treated with 0.001 M DNP

Respiratory quotient (RQ) gives an over-all indication of the synthetic and degradation processes that make up respiration, and might give some clue to the processes taking place in the diseased embryos. The RQ was determined both by the one flask method with KOH and by the two flask method of Dickens and Simer (3) using triplicate samples and three experimental periods. The agreement between the two methods was good. These results (table II) support the hypothesis that fat was being synthesized only in the healthy embryos. The low RQ of the diseased tissues implied utilization of fat as respiratory substrate rather than a synthesis of fat. While the low RQ of the infected embryo might also indicate synthesis of acids, none of these could be detected either by titration or by buffer curves.

Even heavily infected trees produced some normal fruit. When these were compared with healthy fruit the occasional minor differences were attributed to a more plentiful supply of carbohydrate available to these fruits. Since a smaller number of fruits matured on a tree, more carbohydrate was available for each cherry.

TABLE III

CONCENTRATION OF TOTAL & ORGANIC PHOSPHATE EXTRACTED BY 5 % TRICHOLORACETIC ACID FROM BING CHERRY EMBRYOS 18 HOURS AFTER TREATMENT WITH DNP BY VACUUM INFILTRATION.

		Mg TOTAL PO_4/g TISSUE	% ORGANIC PO_4
Healthy	Control	2.32	70
"	0.0005 M DNP	2.27	71
"	0.001 M DNP	2.19	69
Diseased	Control	2.13	45
"	0.0005 M DNP	2.11	42
"	0.001 M DNP	2.03	41
Healthy	Control	2.72	74
"	0.0005 M DNP	2.78	74
"	0.001 M DNP	2.76	74
Diseased	Control	2.70	42
"	0.0005 M DNP	2.73	42
"	0.001 M DNP	2.86	44

When the phosphate in the organic form is expressed as the percentage of total phosphate extracted with 0.75 N trichloroacetic acid from these embryos, the diseased embryos were found to have only about 60 % as much organic phosphate as the healthy. Data from typical experiments are given in table III. Paper chromatography of these extracts indicated that, from a qualitative point of view, glucose-1-phosphate was practically absent from the extract of the diseased tissue.

If the low respiratory rate of the diseased embryos were related to phosphate fixation, as indicated by the low content of organic phosphate, 2,4-dinitrophenol (DNP) might enhance respiratory rate. Loomis and Lipman (5) originally reported the now generally accepted view that DNP uncouples phosphorylation from respiration. At low concentration DNP causes a rise in the rate of oxygen uptake, but at high concentration it depresses the rate. In figure 1a are the results that are obtained when the embryos of Bing cherries just starting to ripen are treated with 0.001 M DNP by vacuum infiltration compared with similar embryos treated with distilled water. In figures 1b and 1c are the same on half ripe and fully ripe cherries, respectively. This concentration of DNP was chosen by trial for this particular experiment. Almost identical results would have been obtained with 0.0005 M or 0.0015 M. DNP gave a moderate increase in rate of oxygen uptake of healthy embryos, but a much greater increase with diseased. The two became approximately the same, although the diseased were usually slightly greater. The DNP, uncoupling phosphorylation, allows oxygen uptake of the diseased tissue to proceed at a rate comparable to that of healthy tissue. This would suggest that the virus affects respiration through the medium of phosphorylation.

The response of these embryos to a number of respiratory inhibitors, including iodoacetate, sodium azide, fluoride, fluoroacetate, and cyanide used singly or in combination was ascertained without giving any additional information. In general, the diseased and healthy embryos responded nearly alike.

DISCUSSION

The literature contains many references to virus effects on metabolism of the host plant. The most usual finding is an increase in the rate of respiration immediately after infection, followed by a rate that is usually lower than that of uninfected tissue. Since this virus produces no visible early symptoms, these experiments are concerned with the late effects only. The lower rate of respiration of the diseased embryos is consistent with the behavior of other viruses.

Blodgett (2), Rawlins (7), and Schneider (9) have found no pronounced effect of the virus on the vegetative growth of cherry trees except where the virus renders the graft union incompatible. The rates of respiration found for such tissues in these

studies are consistent with these findings. In the case of the sour cherry variety, Montmorency, the higher rate of respiration of the diseased leaves might be a direct effect of the virus on the leaves or on the rootstock, but more probably could be attributed to smaller leaves having smaller cells or less admixture of non-respiring cell wall material.

The low rate of respiration of these embryos might indicate that the virus had damaged the respiratory system. The reversal of this virus inhibition by DNP indicates that either phosphate fixation or phosphate utilization is the site of the damage. The former appears, at least superficially, to be the more attractive hypothesis, but these data do not necessarily support either hypothesis. These effects are probably due to coupling of the virus similar to that found by Wood (11) for tumor tissue.

Another possible explanation might be that the virus ties up or competes for the nucleotides that are necessary for phosphate fixation or transfer. This might deprive the synthetic mechanisms of an energy source and prevent the fruit from developing.

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PHYSIOLOGICAL EFFECTS OF GIBBERELLIC ACID. III. OBSERVATIONS ON ITS MODE OF ACTION ON BARLEY ENDOSPERM [1]

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Perhaps the most striking aspect of the normal germination of barley is the correlation between the simultaneous breakdown of reserve carbohydrates and proteins in the endosperm and the growth of the embryo. During germination there are large increases in the activity of hydrolytic enzymes such as the amylases, proteinases, and cytases in the endosperm. The liberation of sugars and amino acids has been correlated with the changes in enzyme activity. On these grounds, it has been suggested that hydrolytic enzymes are responsible for mobilizing the endospermic reserves. The identity is known of some, though by no means all, of the hydrolytic enzymes that may be involved. However, it is not known whether the processes involved with the hydrolysis of starch and the hydrolysis of protein are activated separately or whether they are initially controlled by a common reaction.

Sachs, in 1887 (10) recognized the necessity for embryo action before the hydrolytic processes can begin. James, in 1953, restated the original concept when he wrote, "The relationship of the germinating embryo to the endosperm is virtually that of a parasite extruding exo-enzymes into the host tissues, much as a spider treats a fly" (3). This theory was based almost entirely on the fact that the endosperm exhibits a very low rate of respiration during germination. Since some enzymes, notably α-amylase, are found only after germination, James assumed that only enzyme synthesis could account for their presence and that the endosperm, with its very low respiratory rate, could not be the seat of such synthesis.

There is now reason to believe, however, that this explanation may not describe all of the processes involved. Gibberellic acid (GA_3) is capable (in the excised endosperm in the complete absence of the embryo) of activating starch hydrolyzing enzymes, including α-amylase (9), and releasing reducing sugars (i.e. mobilizing the starch reserves) (8). On the basis of these results it was tentatively suggested (9) that a gibberellin-like hormone may act in vivo in a similar manner, being produced in the embryo and secreted into the endosperm where the hormone would bring about effects similar to those described. In other words, the interdependence between barley embryo growth and the hydrolysis of endospermic reserves would be maintained. The embryo would supply the stimulus or controlling agent in the form of an endogenous gibberellin-like hormone and the endosperm would supply the energy sources necessary for continued growth and development.

The processes occurring in the endosperm during germination, however, involve all of the endosperm constituents and not merely those involved with carbohydrate metabolism. This fact prompted an investigation into the further aspects of the GA_3 effect on excised endosperm.

METHODS

Sterilization, incubation, and estimation techniques have been described in detail previously (7, 8, 9). Briefly, seeds of *Hordeum distichon* L. var. Prior, which had been stored over a saturated $CaCl_2$ solution were used. The seeds were sterilized for 20 minutes with 5% CaOCl, rinsed, hand-husked, resterilized for an hour, cut in half transversely (discarding the embryo half), weighed and placed, 8 at a time, into 5 cm petri dishes. All petri dishes contained 500 γ streptomycin sulfate, were buffered to pH 5.0 with 0.001 M acetate, and made up to a final volume of 3 ml. These were incubated for 22 hours at 24 C. Reducing sugar determinations were performed with the Somogyi reagent (11); protein nitrogen was estimated by the Lowry (5) procedure. Both estimations were carried out on the ambient solutions. Neither *p*-chloromercuriphenylsulfonic acid nor iodoacetate, at the concentrations employed, influenced these reactions, although it was necessary to employ a correction factor due to the interference of streptomycin in the Lowry and Kjeldahl tests.

The moisture content of seeds following sterilization had previously been found to be 11.6% (8); this figure was used as a correction factor. Weights (determined after sterilization) of the groups of eight endosperm in each treatment (in duplicate) were adjusted for the 11.6% moisture content to obtain an initial dry weight figure. This was used for converting all results to a gram dry weight basis. Following the 22 hour incubation period, the groups of eight endosperm were removed, blotted, weighed to determine their fresh weight, and dried to a constant final dry weight in an 80 C forced draft oven. The dry weight loss was obtained by subtracting the final

[1] Received March 31, 1961.

dry weight from the initial dry weight. Water uptake figures were obtained by the following formula:

$$\frac{(\text{Fresh wt} + \text{dry wt loss} - \text{wt following sterilization})}{\text{Initial dry wt}}$$

When seeds were subjected to 100 C dry heat pretreatment before sterilization, separate moisture content determinations were made and used in place of the 11.6 % factor.

The role of microbial action in these results has been examined in the following ways; A: treated endosperm were incubated in the presence and absence of streptomycin, B: soaking periods in CaOCl were extended up to 5 hours, C: GA_3-treated endosperm were transferred to, and incubated on, nutrient agar, and D: respiratory measurements were made during GA_3 treatment. The first two approaches indicated no differences in sugar liberation during the first 24 hours due to variations in these techniques. Visual examination of the cultures on agar indicated the absence of fungal contamination. The respiratory measurements were extended over 48 hours and gave no evidence of infection during this time. Further supporting evidence is the fact that responses occurred in the presence of 10^{-4} M Hg^{++}. Although it is not likely that bacteria are absent from these tests it is very probable that they exert no effect during the short term (1–3 day) experiments reported here.

The GA_3 stock solution was freshly prepared for each experiment. All petri dishes were acid-washed; doubly glass distilled water was used for all solutions.

Results & Discussion

Preliminary experiments demonstrated an increase in the amount of protein nitrogen liberated by endosperm treated with increasing concentrations of GA_3. The reducing sugar and protein nitrogen release, as well as a loss in total dry weight of the endosperm, are illustrated in figure 1. The curves represent the average of six experiments; the difference in ordinate scale for each curve should be noted. As the concentration of GA_3 increases, a concomitant increase in the magnitude of each effect can be noted, until an optimal concentration of 2 γ (2×10^{-6} M) is reached. The 2 and 200 γ values are not significantly different from one another, but the other sugar and nitrogen values differ at the 0.001 significance level while the dry weight values differ significantly at the 0.01 level. The protein nitrogen (presented here as nitrogen rather than protein) and the sugar values, taken together, represent 8, 14, 15, and 16 % of the dry weight loss respectively at 0, 0.02, 2, and 200 γ GA_3. If the nitrogen figures are converted to protein ($\times$ 6.25) the percentages are 13, 20, 23, and 24, respectively. The sugar concentrations are only approximate values because they are calculated as glucose. (Maltose, which is undoubtedly present (8) exhibits only half the absorbance of glucose in the Somogyi test; as a result, the proportion of maltose in the assay would be accorded only half its weight equivalent.) GA_3 seems capable not only of increasing the magnitude of all three functions, but also of changing the proportion of the dry weight loss which

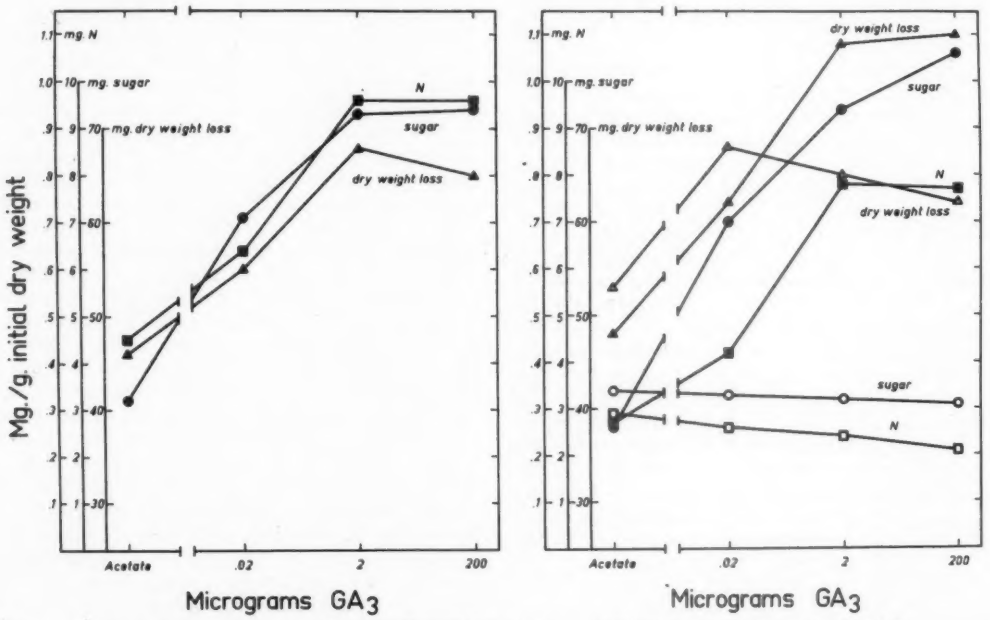


Fig. 1. Endosperm responses to various concentrations of GA_3. Incubation temperature equals 24 C.

Fig. 2. Effect of prior heat treatment (100 C for 1 hr) of intact grain on subsequent GA_3-induced responses of excised endosperm; *closed symbols*—unheated; *open symbols*—heated.

is reflected as sugar and nitrogen. This change, which remains fairly constant under the conditions of the experiment, seems to depend more on the presence of GA_3 than on its concentration.

Figure 2 illustrates that the three characteristics of the GA_3 treatment are suppressed by prior exposure of the intact grain to heat (100 C for 1 hr). Difficulty has been encountered in measuring the dry weight loss since each point on the curve is dependent upon the averages of two sets of six weighings. This is reflected, as well, in the lesser significance level of the difference between dry weight values of figure 1. Also, although sugar and nitrogen results are little influenced by changes in initial moisture content, the dry weight loss curve can be caused to shift, in this instance, 11 mg, by a 1 % error (i.e. if the initial moisture content is 13 % instead of 14 %) in the moisture content determination. A shift of this nature does not affect the absolute relationships between the points of any one curve.

Since the Lowry test may be influenced by factors other than protein, total Kjeldahl nitrogen determinations were made and the results of both estimations, as well as the non-protein nitrogen fraction (derived by difference) are presented in figure 3. The total nitrogen curve closely approximates the Lowry results whereas the non-protein nitrogen curve shows little or no effect of increasing GA_3 concentrations. To ascertain whether or not the increase in sugar exerts an effect on the Lowry test, various amounts of sucrose, fructose, maltose, and glucose were added to control assays. Only a concentration of fructose, many times that observed in the incubates (8) interfered. To check for the presence of non-protein phenolic groups, etc., the Lowry reagent was altered by leaving out the copper. The results indicated a minimum of extraneous material. Finally, the contents of the ambient solutions of many duplicate petri dishes were combined, dialyzed, and chromatographed on a DEAE-cellulose column. Dialysis did not decrease the difference in nitrogen between + and — GA_3 treatments, and the proteins present were similar to the protein components of extracts of ground endosperm (Paleg & Simmonds, unpublished data). It seems probable, therefore, that the majority of the Lowry reaction is, in this case, a function of protein. Peptides, however, also contribute to the Lowry reaction and it is probable that a portion of the protein values may be due to these components.

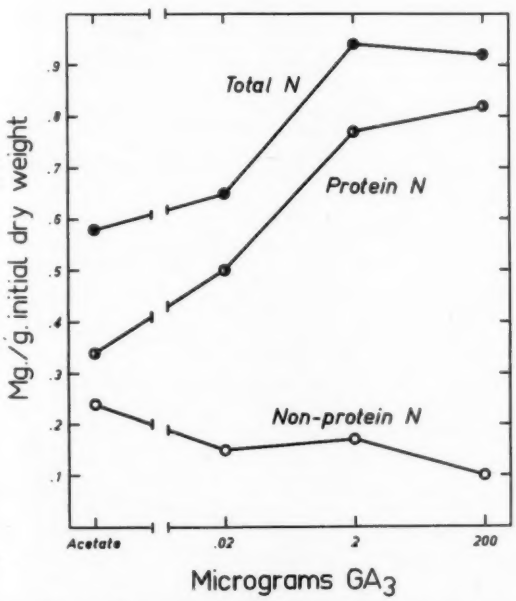
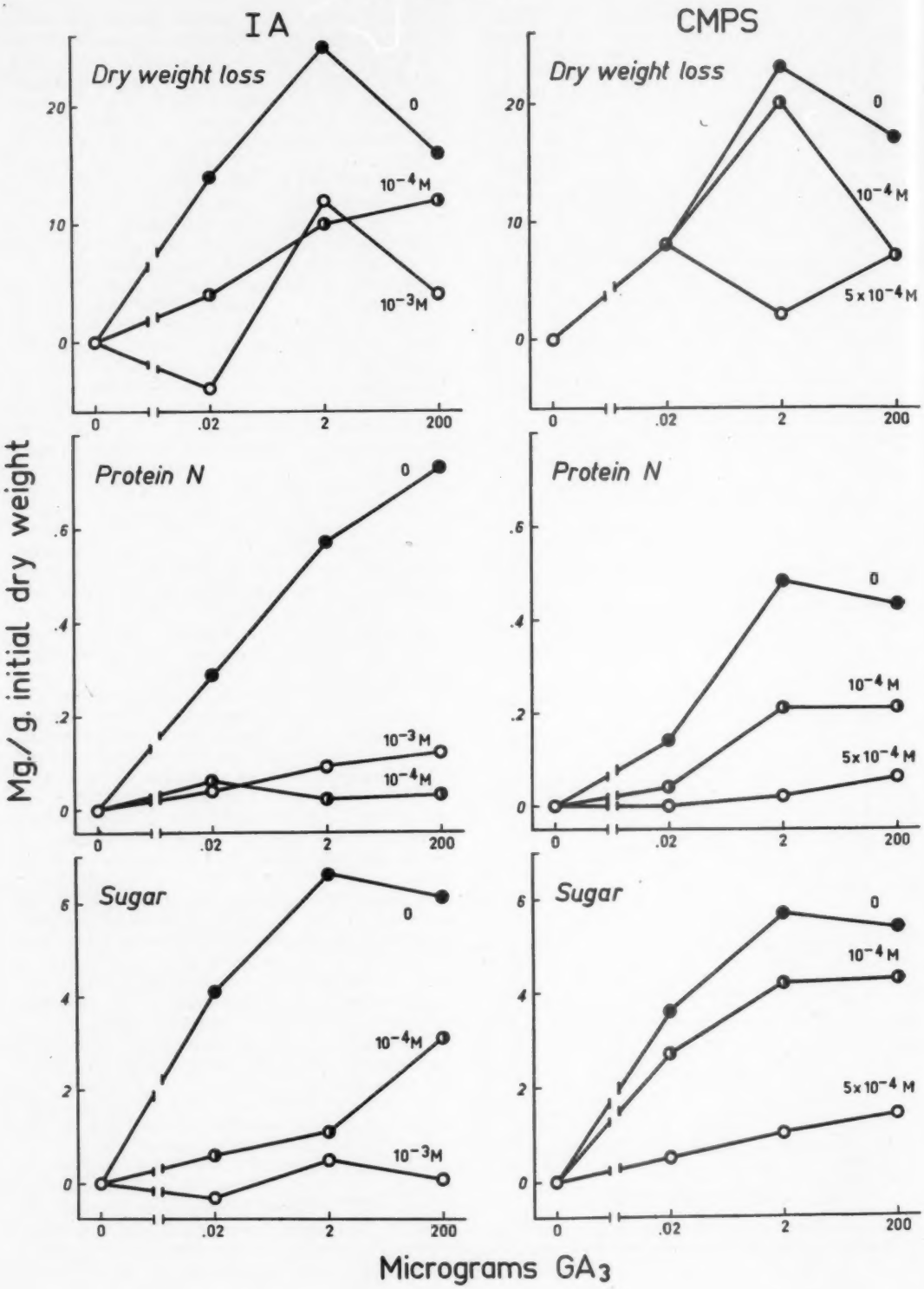


FIG. 3. Nitrogen-containing fractions released by endosperm as a response to various concentrations of GA_3 (non-protein nitrogen curve derived by difference).

Attempts were made both to inhibit and separate the GA_3 effects. Preliminary experiments indicated that $HgCl_2$ at a concentration of 10^{-3} M would completely eliminate all three responses; figures 4 and 5 illustrate the effects of iodoacetic acid (IA) and *p*-chloromercuriphenylsulfonic acid (CMPS). These compounds are protein precipitants as well as inhibitors of sulfhydryl-containing enzymes. Figures 4 and 5, plotted as the inhibition of the GA_3 effect (using the acetate control values as zero), indicate that a concentration of 10^{-3} M for either compound is, or would be, sufficient to completely inhibit all three responses of the endosperm. The curves also demonstrate that the three effects of GA_3 are increasingly inhibited by raising the inhibitor concentration and would suggest an association of the responses with sulfhydryl-containing compounds. The absolute values indicated that even at the higher levels of the inhibitors, as with the effect of the heat treatment (fig 2), the endosperm responses did not fall appreciably below those of the acetate controls. This is evidence that GA_3 is inducing the initiation of new processes, rather than enhancing the processes concerned with the responses of the endosperm in the absence of GA_3.

Figure 6 illustrates the lack of effect of GA_3 on water uptake over a temperature range of 10 to 40 C. Results comparable to these were obtained with other treatments, and no consistent trend in the effects of GA_3 on water uptake was discernible. This supports the results of other workers (6) who also found no effect of GA_3 on water uptake of seeds.

The results of varying the incubation temperature on sugar and protein nitrogen release and dry weight loss are presented in figure 7. The optimum temperature for all three reactions is 30 C. The curves of the responses in the absence of GA_3 show no temperature-controlled inflection, evidencing only a very gradual increase as the incubation temperature increases. In this experiment the position and shape of the $-GA_3$ nitrogen release curve gives the impression that there is no effect of GA_3 on nitrogen release at temperatures lower than 30 C. As we have seen (fig 1), this is probably not so.


IA
CMPS
Dry weight loss
Protein N
Sugar
0
10⁻⁴M
10⁻³M
5x10⁻⁴M
Mg./g. initial dry weight
Micrograms GA3
0
.02
2
200

An attempt was made to check three points; A: the effect of prior heat on the dry weight loss of endosperm in the presence of GA_3 (fig 2); B: the placement of the nitrogen release curve in the absence of GA_3 (fig 7), and C: the response of the endosperm, in the presence of GA_3, to a temperature of 30 C (fig 7). Grain were divided into two groups, one of which received prior heat treatment (100 C for 1 hr), before being subjected to the usual sterilization procedure. The heat-treated group was incubated at four temperatures with or without 2γ GA_3 while the non-heated group was incubated at the same temperatures with GA_3. The results (fig 8) confirm the observation that heated grain does not respond, within the time of treatment, to the presence of GA_3 at any of the temperatures. Evident, as well, is the fact that the response of heated endosperm in the presence and absence of GA_3 is identical; by comparison with figure 7, it can be seen to be also identical with non-heat-treated endosperm in the absence of GA_3. The two nitrogen release curves of heated endosperm (fig 8) indicate that the curve of nitrogen release in the absence of GA_3 in figure 7 is probably displaced, while the results obtained with the unheated, GA_3-treated endosperm of figure 8 amply confirm the endosperm's marked responsiveness to temperature noted in figure 7.

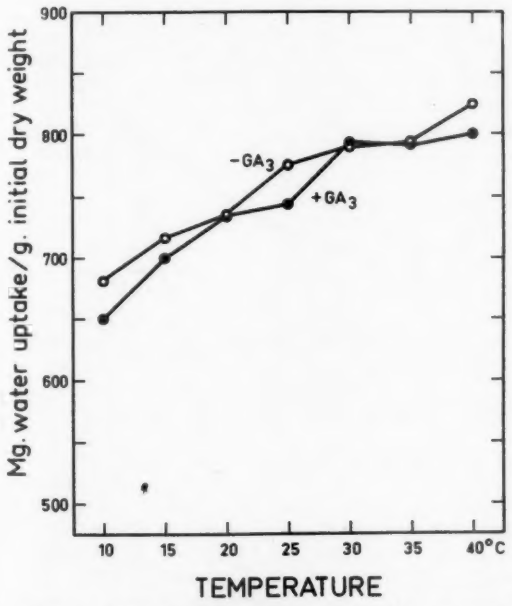


FIG. 6. Lack of effect of 2×10^{-6} M GA_3 on water uptake of endosperm subjected to various incubation temperatures.

TABLE I

EFFECT OF GA_3 ON PERCENT OF DRY WEIGHT LOSS IDENTIFIED AS REDUCING SUGAR & PROTEIN N AT VARIOUS TEMPERATURES

Temp °C	% of dry wt loss	
	$+GA_3$	$-GA_3$
10	5.1	5.4
15	6.2	5.4
20	10.9	7.6
25	17.5	7.9
30	28.4	9.9
35	26.8	10.8
40	8.2	9.9

To exclude the possibility that a temperature of 40 C might cause inactivation of the GA_3, 0.1 ml portions of the ambient solutions of the 10 C and 40 C treatments after incubation were applied to dwarf corn plants (d–1) as used in gibberellin bioassay tests (1). Six plants were treated with each solution; the average growth due to GA_3 of the two groups was identical. This provides good evidence that the differences shown in figures 7 and 8 at 35 and 40 C are not due to the destruction of the applied GA_3 by temperature, but are due to the interaction of the effects of temperature and GA_3 on the endosperm.

The results of incubating at 40 C indicate that whether or not GA_3 is present, the values are about the same. This suggests that an incubation temperature of 40 C prevents, rather than inactivates, whatever processes are initiated by GA_3 at lower temperatures. In this regard, it is interesting that Sachs, as reported by Hopkins and Krause (2), noted that germination of barley will not take place at temperatures above 38 C.

If the sugar and nitrogen values in the presence of GA_3 are calculated as percent of dry weight loss (table I) then as the temperature increases, reducing sugar and protein nitrogen values also increase, reaching a maximum at 30 C. As was seen from the discussion of figure 1, changes in the concentration of GA_3 do not seem to cause changes in this relationship. In other words, at any one temperature the GA_3-initiated responses probably occur at a constant rate (with respect to one another) dependent upon

FIG. 4 (*left*). Effect of iodoacetic acid (IA) on the GA_3-induced dry weight loss, protein nitrogen release, and reducing sugar release. (Points obtained by subtraction of acetate control values from GA_3 values).

FIG. 5 (*right*). Effect of *p*-chloromercuriphenylsulfonic acid (CMPS) on the GA_3-induced dry weight loss, protein nitrogen release, and reducing sugar release. (Points obtained by subtraction of acetate control values from GA_3 values).

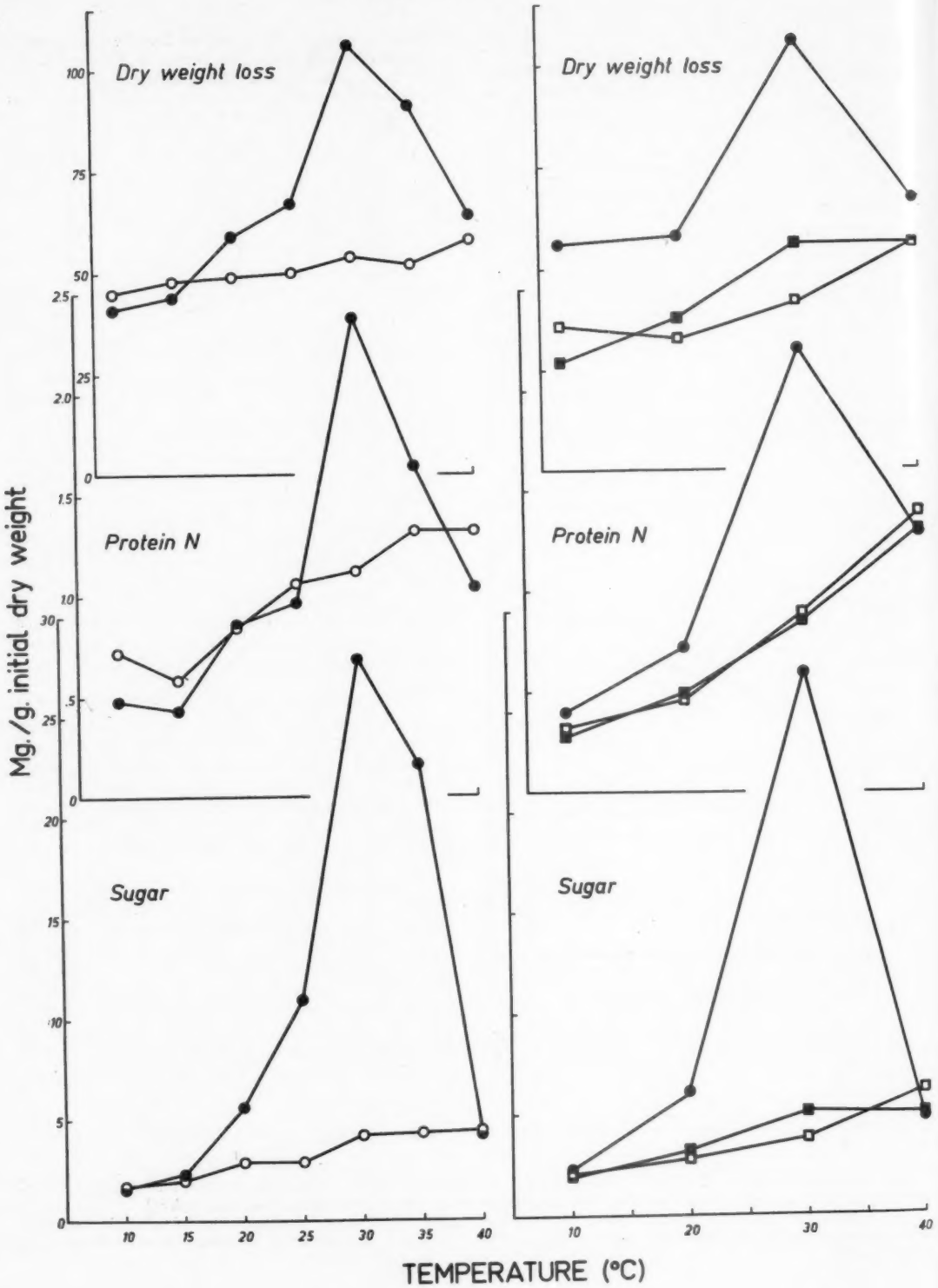

Dry weight loss
Protein N
Sugar
Mg./g. initial dry weight
TEMPERATURE (°C)

TABLE II

Q10 VALUES FOR SUGAR, N, & DRY WEIGHT LOSS AT 20 & 30 C IN PRESENCE & ABSENCE OF GA_3

Temp	SUGAR			PROTEIN N			DRY WT LOSS		
	$-GA_3$	$+GA_3$	DIFF.	$-GA_3$	$+GA_3$	DIFF.	$-GA_3$	$+GA_3$	DIFF.
				Figure 7					
20 C	2.9	5.6	2.7	0.84(0.60)	0.85	0.01(0.25)	49	59	10
30 C	4.2	27.8	23.6	1.12(0.88)	2.38	1.26(1.50)	54	106	52
Q10	1.5	5.0	8.7	1.3(1.5)	2.8	? (6.0)	1.1	1.8	5.2
				Figure 8					
				(*Avg. of heat-treated grain taken as* $-GA_3$)					
20 C	3.0	6.1	3.1	0.48	0.72	0.24	36	58	22
30 C	4.4	26.9	22.5	0.87	2.20	1.33	49	106	57
Q10	1.5	4.4	7.3	1.8	3.1	5.5	1.4	1.8	2.6

the concentration of GA_3. However, the changes in the relationship between sugar and protein nitrogen, and dry weight loss shown at various temperatures provides the first evidence that the responses can be influenced independently of one another, even though they may all be initiated as a common effect of GA_3.

In table II are presented values (20 & 30 C) from figures 7 and 8 and the Q10 figures derived from them. In calculating the Q10 figures from figure 8 the heat-treated endosperm results were averaged and taken as the $-GA_3$ treatment. The values from figure 7 in parentheses (protein N) were derived by adjusting the $-GA_3$ curve to equality with the $+GA_3$ curve at 10 C, and are included for comparison. The Q10 figures for the $-GA_3$ treatments in all instances are below 1.8, averaging about 1.4. This is approximately the magnitude expected if the temperature-induced responses in the absence of GA_3 were the result of a physical phenomenon, i.e., diffusion. On this assumption, to obtain the Q10 of the GA_3 effects, the $+GA_3$ values should be corrected for the results obtained in the absence of GA_3. The corrected results are recorded in the columns headed "Difference".

There is good agreement between the values in the two experiments except for the Q10, derived by difference, of the dry weight loss. The magnitude of the Q10 for sugar release (absolute or corrected) and protein nitrogen release (corrected) are very high (i.e., 4.4–8.7), well above the range of Q10 values for single enzyme actions (i.e., 2–3). These results suggest increases in amounts of enzyme as well as enzyme activity. The dry weight loss results are not as clear but enzyme production and action are probably important in this temperature-dependent GA_3-induced response as well. These results are in accord with the demonstration of enzyme activation reported previously (9).

To ascertain the extent of the effects of GA_3 on excised endosperm at one temperature, 24 C, measurements of the endosperm responses were taken over a 3 day period. Individual petri dishes were used for sampling at each time so as to avoid dilution effects. The times indicated in figure 9 refer to the period the endosperm were in contact with the solutions, all of which contained acetate buffer (0.001 M-pH 5.0), 500 γ streptomycin and 2 γ GA_3 per petri dish.

Figure 9 indicates the results obtained when sugar and nitrogen release and dry weight loss, in the presence and absence of GA_3, are plotted against time. The dry weight loss curve in the absence of GA_3 shows a gradual increase over the 3 day interval. In the presence of GA_3 the slope of this curve is greatly increased; by the end of the 3rd day, the endosperm have lost almost half their initial dry weight. It seems clear that a loss in dry weight of this magnitude must involve all parts of the endosperm. Sugar and protein values also show a marked increase, with time, in the presence of GA_3. One-third of the initial dry weight of the endosperm (or about 60 % of the dry weight loss) has been converted to reducing sugar. This represents a 1000-fold increase over the amount of reducing sugar present in the ambient solution initially. Nitrogen released is also high although not as great as the sugar, being of the order of a 100-fold increase. In the absence of GA_3, the increases are 100-fold and 20-fold, respectively.

Experiments of this type have been performed at 30 C as well; the reaction rates of all three responses are increased considerably, reaching the stage

FIG. 7 (*left*). Endosperm responses to various incubation temperatures in the presence (●) and absence (○) of 2×10^{-6} M GA_3.

FIG. 8 (*right*). Effect of prior heat treatment (100 C for 1 hr) on endosperm responses at various incubation temperatures. Unheated with GA_3 (●): heated with GA_3 (■): heated without GA_3 (□). GA_3 concentration equals 2×10^{-6} M.

indicated at 3 days in figure 9 after only 2 days at the higher temperature.

The results also indicate that changes take place in the absence of added GA_3 although to a greatly reduced extent. This is not surprising since it must be recalled (9) that some β-amylase and possibly even some α-amylase (albeit a very small amount) are present and active in the untreated endosperm. Small and variable amounts of proteinases and cytases which might be involved are probably also present and active, although it is extremely unlikely that, even over extended periods of time, their activity would produce effects similar to those induced by the presence of GA_3. Another interpretation of the results is also feasible. It has not been possible to determine if these responses would take place in the complete absence of a gibberellin since it is probable that a small amount of endogenous gibberellin is already present in the endosperm. The results recorded here in the absence of exogenous gibberellin may represent the effects of an endogenous gibberellin. If present, it is in such small amount as to cause only an indication of its presence without being able to effectively activate all of the hydrolytic processes involved in the dissolution of the endosperm.

General Conclusions

The reactions of the barley endosperm to applied GA_3 appear to involve all the major components, starch, protein, and cell walls. Treatment of excised endosperm with GA_3 induces a release of protein nitrogen and reducing sugar into the ambient solution as well as a loss in dry weight of the endosperm. If the time of the experiment is extended to 3 days, these changes assume major proportions; 50 % of the dry weight of the endosperm is lost with at least 80 % of this loss recoverable as reducing sugar and protein nitrogen. The remainder of the dry weight loss represents an, as yet, undefined fraction.

Responses of the endosperm to applied GA_3 are temperature-dependent and the Q10 values for these responses are very high. Enzyme activation appears to be the necessary precursor for the other GA_3 effects recorded here, paralleling the action of the embryo on the endosperm during germination. Also, in these experiments, there can be no doubt as to the derivation of the enzymes involved since the embryo was removed at the beginning of each experiment. It appears, therefore, that the barley endosperm can supply the enzymes necessary for its own autolysis and that GA_3 is capable of causing the activation of most if not all of these enzymes. It is not yet known whether the GA_3 response syndrome is identical with or merely similar to the changes that occur in the endosperm during germination. Qualitatively, the results strongly suggest such an identification. In this connection Lazer et al. (4) recently reported the presence of both GA_3 and a precursor in malting barley, the concentration of GA_3 increasing fourfold during malting.

It has not yet been possible to separate the GA_3-induced responses of the endosperm. Similarities in effects of prior heat, temperature, and inhibitor action all suggest that GA_3 is exerting one major, trigger effect and that this is the limiting reaction. Differences between the Q10 values of the three endosperm responses, however, imply that, once initiated, the

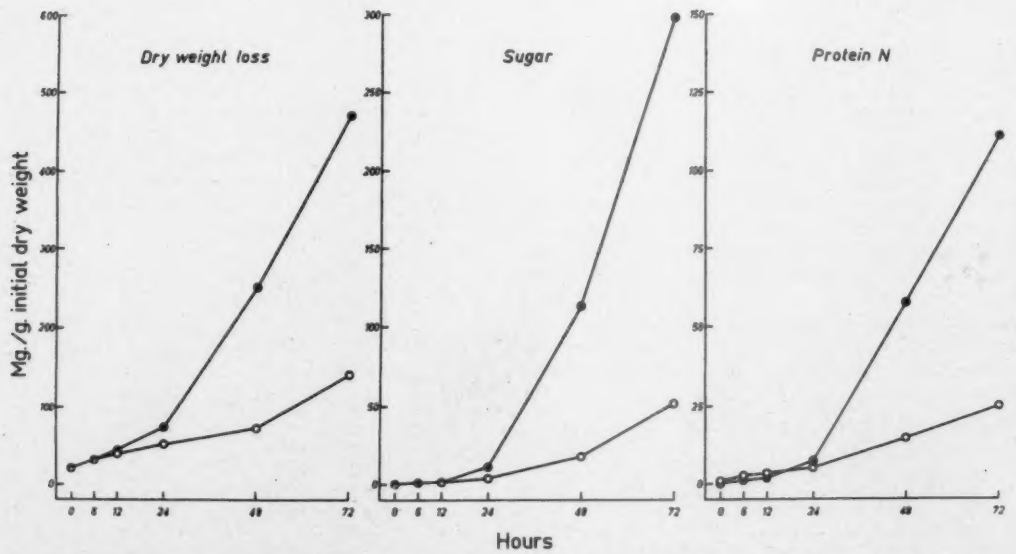
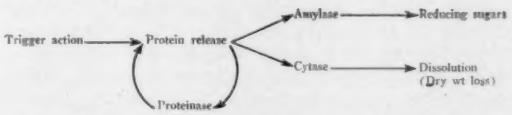


Fig. 9. Endosperm responses at various incubation times in the presence (●) and absence (○) of 2×10^{-6} M GA_3.

processes are probably controlled by different mechanisms.

The production of reducing sugars is probably no more than a quarternary manifestation of GA_3 action. Activation of amylolytic enzymes, shown previously (9), must precede sugar liberation and could, therefore, represent a tertiary effect of GA_3. In a sequence such as this, the theoretical secondary effect may be the general release of protein, of which the amylases are but one evidence. The action of GA_3 enabling protein concentration in solution to increase might be the initial trigger or hormonal mechanism of action. The following diagram illustrates these relationships:



The sequence indicated above is, at best, an attempt to relate the various manifestations of GA_3 action. The scheme, which may represent an oversimplification, in fact, is not the only one consistent with the available information.

The respiration rate of mature endosperm tissue is low even during germination when sugars are plentiful. This suggests that synthetic processes are at a minimum. It also indicates that the GA_3 effects on endosperm, particularly those involving an increase in protein concentration in solution, may not be explicable in terms of an influence on synthetic systems. From this viewpoint it is particularly difficult to relate the GA_3 effects reported here with those described for actively growing tissues or whole plants. It is possible, however, that some hormonal effects in plants may be brought about by a type reaction such as a change in the physical properties of a particular class of compounds. This, in one tissue, might manifest itself as a change in the permeability characteristics of a membrane, and in another tissue, result in the activation of enzymes.

Summary

As a response to increasing amounts of GA_3 the barley endosperm liberates increasing quantities of reducing sugar and protein. There is, as well, a proportional decrease in the resultant dry weight of the endosperm. This GA_3-induced response syndrome can be prevented by preheating the intact grain before treatment with GA_3, or by incorporating one of several sulfhydryl reacting compounds in the incubation solution. The response syndrome over the first 24 hour period is also sensitive to incubation temperature, evidencing a pronounced maximum at 30 C. Q10 values derived from the endosperm responses to GA_3 range from 2.6 to 8.7 indicating that enzyme formation or activation is occurring. When the incubation time, at 24 C, is extended to 3 days half the initial dry weight of the endosperm is lost to the ambient solution with a large proportion of this loss being recoverable as reducing sugar and protein. A possible sequence of events leading to these manifestations is discussed, and a tentative hormonal role for gibberellin, in the germination of barley, is suggested.

Acknowledgment

The assistance of Miss Jan Sincock is gratefully acknowledged, and the author is also indebted to Mr. A. Jennings for his excellent technical help.

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FORMATION OF AUXIN FROM TRYPTOPHAN THROUGH ACTION OF POLYPHENOLS[1,2]

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Plant preparations that convert tryptophan to indoleacetic acid (IAA) characteristically have low reaction yields. Since such preparations sometimes also contain IAA-oxidase activity, inhibition of the oxidase might raise the yields of IAA. Accordingly, polyphenolic inhibitors (1, 11, 19, 33, 40) were added to incubation mixtures containing tryptophan and a seedling enzyme preparation that was able to convert tryptophan to IAA. More than tenfold increases in auxin yield resulted. However, the presence of auxin in enzymeless controls, plus experiments on the recovery of added IAA, indicated that the enhancements observed might be caused by a polyphenolic oxidation of tryptophan to IAA. We shall present evidence that the sequence

$$\text{Phenols} \xrightarrow[\text{Phenolase}]{O_2} \text{Quinones}$$

Quinone + Tryptophan → Indolepyruvic acid → IAA

occurs in plant material, and describe several of its characteristics.

METHODS

ENZYME PREPARATION: Mung bean seedlings (*Phaseolus aureus*, Roxb.), 7 to 8 days old, were the usual source of enzyme. The plants were grown in gravel periodically flushed with tap water. An 18-hour day at 25 C was alternated with a 6-hour night at 18 C. Illumination came from a mixture of 4500°K-fluorescent tubes and tungsten lamps in a wattage ratio of 5:1. This supplied an irradiance of 2,500 ft-c at plant level.

Lyophilized cell-free extracts were used unless otherwise noted. Leaves and buds, 2½ kg, in 500 g portions, were frozen in liquid N_2. A cold-room was used for subsequent operation. One of the frozen portions was dispersed in 0.2 M tris(hydroxymethyl) aminomethane buffer, pH 7.2, with a colloid mill. The dispersion was clarified by centrifugation through sharkskin paper in a basket head. Thereupon an additional 500 g of tissue was dispersed in the filtrate and recentrifuged. The same procedure was repeated until all of the 2.5 kg were processed. Each filtrate was kept above pH 6.8 by adding N NaOH as required. About 900 ml of the final filtrate were centrifuged 4 hours at 44,000 × *g* to yield a supernatant fraction containing 42 mg/ml of solids and 1.2 mg/ml of nitrogen precipitated by trichloroacetic acid (TCA). The total volume was adjusted to pH 7.2, lyophilized, and stored in vacuo over P_2O_5 at 2 C. For use, the dry powder was dissolved in distilled water at a concentration of 42 mg per ml.

Fresh enzyme preparations were made by freezing tissues in liquid N_2 and grinding them in a mortar with twice their weight of phosphate buffer, 0.1 M, pH 7.2. The slurry was filtered through nylon cloth and centrifuged at 105,000 × *g* for 30 minutes. The supernatant liquid was adjusted to pH 7.2 and used immediately; 3 ml were added to each incubation flask.

INCUBATION & EXTRACTION PROCEDURE: Unless otherwise noted, the incubation mixture consisted of 0.02 M L-tryptophan, 42 mg of enzyme, 0.03 M tris buffer, and catechol at 0.01 M in a total volume of 10 ml. Solutions of the various components were adjusted to pH 7.2 just before use. The tryptophan (Mann) was previously extracted with diethyl ether for 1 day in a Soxhlet apparatus to remove ether-soluble indolyl impurities.

Incubation mixtures, in 25-ml Erlenmeyer flasks, were placed in a Dubnoff shaker at 30 C and agitated in the dark at a frequency of one cycle per second for 1 hour. A zero-time sample, taken immediately after mixture of the components, was used as a base for the zero-order reaction rates represented by the data. Reactions were halted by rapidly bringing the contents of a flask to boiling point with a bunsen flame. On cooling, the pH was adjusted to 9.2 with NaOH. Neutral and basic fractions were removed with diethyl ether, as was the acid fraction after adjusting the aqueous phase to pH 3 with HCl. Suspended water was precipitated from the ether with the acid fraction by storage at −20 C for several hours. The ether was then decanted and removed from the extract by gentle boiling over a steam bath.

All of the diethyl ether used was purified by distillation from aqueous CaO-$FeSO_4$ (28). Phenolic solutions were freshly prepared. Commercial reagents were used throughout with the exception of 3-nitrocatechol, which was first purified by sublimation in vacuo, mp 59-69 C.

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[2] This work was performed under the auspices of the U.S. Atomic Energy Commission.

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AUXIN ASSAY: The acid fraction was taken up in a small quantity of methanol previously purified by distillation from Zn-KOH, and a portion was resolved by paper chromatography (cf. 16 for details). Following ascending chromatography in *n*-butanol: 28 % ammonia: water, 10:1:1 v/v/v, IAA, as indicated by parallel marker strips, was eluted with methanol and estimated colorimetrically with the iron-perchloric acid reagent (ibid). Less than 2 μg IAA in heterogeneous extracts cannot be assayed satisfactorily by the above procedure. The term "tr." is used in the data to indicate that a faint trace of color was seen at the R_f of IAA on marker chromatograms. No IAA was detected in the various appropriate controls of tryptophan alone.

INDOLE-3-ACETALDEHYDE & INDOLE-3-PYRUVIC ACID: Indoleacetaldehyde was synthesized following the procedure of Gray (20), and stored as the bisulfite-addition complex. Free indoleacetaldehyde was liberated from an aqueous solution of the bisulfite complex by adding Na_2CO_3, and extracted with ether. The aldehyde was transferred to added water by evaporation of the ether.

Indolepyruvic acid was synthesized by condensing indole-3-aldehyde with hippuric acid to form the acetylated benzoyl azlactone (mp 207–209 C uncor); the azlactone was then hydrolyzed to indolepyruvate (5). The elementary composition was, in percentage: C-65.28 (65.01 theoretical); H-4.66 (4.47); O-23.03, by difference, (23.62); N-7.03 (6.89). Its melting point, with decomposition, was 212.0 to 215.0 C, uncor, when introduced at 200 C, and 211.5 to 214.0 C, uncor, when introduced at 205 C. The *p*-nitrophenylhydrazone melted at 153 to 154° uncor (cf. 10), with darkening and decomposition.

RESULTS

The influence of catechol on the yield of IAA from tryptophan is shown in table I. IAA is formed from tryptophan by the action of catechol without enzyme. However, all four plant preparations produced more auxin in the presence of the phenol. The latter results are not inconsistent with a suppression of IAA inactivation by catechol.

TABLE II

RECOVERY OF IAA ADDED TO ENZYME & CATECHOL MIXTURES

IAA ADDED, μg	% RECOVERY		
	CATECHOL	ENZYME	CATECHOL + ENZYME
500	86	88	81
100	66	84	70
20	35	35	25

To test this explanation, the effect of catechol on the recovery of added IAA was determined. Synthetic IAA, recrystallized as the sodium salt, was added to incubation mixtures. These were assayed for IAA after a 1-hr incubation period. The recoveries obtained are given in table II. The loss of IAA with catechol alone was about the same as that with enzyme alone. Mixture of the catechol and enzyme did not increase the recovery of IAA; actually, a somewhat lower recovery was observed. These results indicate that exposure to enzyme and to catechol caused a significant loss of IAA, and that the enhanced yields of IAA in the presence of catechol (table I) were not caused by an inhibition of an IAA-oxidase action.

An alternative hypothesis appeared compatible with the above data: catechol alone can degrade the amino acid to IAA, a degradation that is enhanced in the presence of the plant enzymes. In the following experiments this hypothesis is elaborated.

EFFECT OF VARIOUS PHENOLS: The activities of several mono- and dihydric phenols in the enzymatic and non-enzymatic conversion of tryptophan to IAA are given in table III. Catechol was the only phenol tested that formed significant amounts of IAA without enzymatic participation. The enzyme was re-

TABLE I

EFFECT OF CATECHOL ON CONVERSION OF TRYPTOPHAN TO IAA BY SEVERAL PLANT PREPARATIONS

	MUNG BEAN		MUNG BEAN		WITHOUT ENZYME	
	−C***	+C***	−C	+C	−C	+C
μg IAA/hr	2.0*	31*	0**	14**	0	10-11
μg IAA/hr/mg N	2.0	31	...	...	...	...
			Oat		*Sunflower*	
μg IAA/hr	1.6†	29†	2.8†	15†	tr.†	36†
μg IAA/hr/mg N	0.7	13	2.2	12	...	22

* Lyophilized.
** Lyophilized, boiled 10 minutes.
*** −C, without catechol; +C, with catechol.
† Non-lyophilized. *Avena sativa*: etiolated coleoptile and primary leaf, age 72 hours. *Helianthus annuus*: terminal leaves and buds from 1-month-old green plants.

TABLE III

TRYPTOPHAN CONVERSION TO IAA IN PRESENCE OF VARIOUS PHENOLS

ADDEND	CONC. M	IAA, % YIELD RELATIVE TO CATECHOL — ENZYME	+ ENZYME
Catechol	10^{-2}	12 μg=100	32 μg=100
3-Nitrocatechol	10^{-2}	tr.	47
Protocatechuic acid	10^{-2}	0	150
Caffeic acid	10^{-3}	tr.	77
Chlorogenic acid	10^{-2}	tr.	100
Tyrosine	10^{-3}	0	0
Dihydroxyphenylalanine	10^{-3}	0	12
Resorcinol	10^{-2}	0	0
Pyrogallol	10^{-2}	...	32
Phenol	10^{-2}	0	73
o-Nitrophenol	10^{-3}	0	0
o-Cresol	10^{-2}	0	4
m-Cresol	10^{-2}	0	29
p-Cresol	10^{-2}	0	98

quired for the activity of the other phenols, and increased the yield from catechol. The ortho-dihydroxy structure seems to be required for appreciable activity, since resorcinol, tyrosine, *o*-nitrophenol and *o*-cresol were ineffective; these compounds also have significantly lower oxidation rates than their *o*-dihydroxy analogs (22, 26). The activity of phenol, pyrogallol, and *m*- and *p*-cresol probably derive from their ready conversion by phenolase containing systems to the vicinal dihydroxy analogs (9, 30).

Quite active in producing IAA were the *para*-substituted protocatechuic, caffeic and chlorogenic acids, *p*-cresol, and, to a lesser extent, dihydroxyphenylalanine. Their effectiveness indicates that the nature of the para substituent is not highly specific, a characteristic consistent with the inference that a *p*-substitution stabilizes the *o*-quinoid structure through repeated oxidations and reduction (39). Apparently the substituent can involve either a carbon or a nitrogen linkage.

INHIBITORS OF CATECHOL-MEDIATED AUXIN FORMATION: Known inhibitors of phenolic reactions were then tested for their effect on auxin formation (table IV). The reducing agents were highly effective in lowering the yield; ascorbic acid and cysteine, which would be expected to act as oxidation inhibitors and to reduce quinones, gave virtually complete inhibition of both the enzymatic and non-enzymatic formation of IAA. The chelating compounds reacting preferentially with iron caused a 15 to 30 % inhibition, though inhibition by phenanthroline in the system without enzyme, where dipyridyl has no effect, suggests that this class of compounds might act in part as competitive reductants. The copper reagent, diethyldithiocarbamate, caused an 85 % reduction in the amount of IAA produced with the enzyme. Interestingly, azide, which apparently inhibits both tyrosinase (24) and laccase (25), had no effect on the yield with or without the enzyme even at a concentration of 10^{-1} M. Tryptophan probably was responsible for the lack of inhibition, since azide did retard the initial rate of catechol oxidation by the enzyme when tryptophan was omitted. (Solutions of catechol & enzyme darkened rapidly, but not in the presence of azide.)

TABLE IV

EFFECT OF REDUCING & CHELATING AGENTS ON IAA YIELD

ADDEND	CONC. M	μg IAA/hr — ENZYME	+ ENZYME
None	...	12	30
Ascorbic acid	10^{-2}	0	3.6
Cysteine	10^{-2}	0	0
Diethyldithiocarbamate	10^{-2}	2.6	5.1
Ethylenediaminetetraacetate*	10^{-2}	2.8	20
o-Phenanthroline	10^{-3}	4.8	24
α,α-Dipyridyl	10^{-3}	12	25
Azide*	10^{-1}	12	36

* Catechol concentration 5×10^{-3} M; 10^{-2} M in all others.

TABLE V

EFFECT OF NITROPHENOLS ON FORMATION OF IAA

ADDEND	CONC. M	RELATIVE YIELD (%) ENZYME + TRYPTOPHAN + CATECHOL*	+ PHENOL*
None	...	30 μg=100	22 μg=100
o-Nitrophenol	10^{-4}	100	...
	10^{-3}	130**	110
	10^{-2}	150	130
p-Nitrophenol	10^{-4}	110	90
	10^{-3}	95	120
2,4-Dinitrophenol	10^{-4}	110	100
	10^{-3}	92	85
	10^{-2}	58	31
3-Nitrocatechol	10^{-2}	110	...

* 10^{-2} M

** Catechol concentration, 10^{-3} M.

EFFECT OF NITROPHENOLS: Mono-nitrophenols inhibit neither the catechol- nor the phenol-mediated formation of IAA. The data in table V indicate that when significant differences are observed, enhancement rather than inhibition of the reaction occurs. As with the lack of azide inhibition, the explanation may lie in the presence of tryptophan. The higher concentrations of 2,4-dinitrophenol significantly decreased the amount of IAA obtained with both catechol and phenol. Though 3-nitrocatechol could partially replace catechol function in the formation of IAA (table III), the presence of nitrocatechol and catechol in equimolar concentrations yielded the same amount of IAA as did catechol alone.

TYROSINASE: From the preceding experiments it appeared that a polyphenolase action was involved in the conversion of tryptophan to IAA. The results

TABLE VI

IAA FORMATION WITH TYROSINASE*

	IAA/μg/hr
Tyrosinase + catechol	0
Tyrosinase + tryptophan	0
Catechol + tryptophan	6
Tyrosinase + catechol + tryptophan	25

* Medium: L-tryptophan 0.02 M, catechol 10^{-2} M, tris buffer 0.025 M, mushroom tyrosinase (Worthington) 0.25 catecholase units, total volume 10 ml, pH 6.5.

obtained when mushroom tyrosinase was used as the enzyme (table VI) support this view. Tryptophan and tyrosinase produced no IAA. When catechol was added, tyrosinase was able to reproduce the action of the mung bean enzyme.

ACTION OF QUINONES: Since the degradation of tryptophan to IAA is an oxidative reaction, the non-enzymatic formation of IAA by catechol suggested that a first step might involve dehydrogenation of the phenol. Preflushing of the solutions with N_2 gas (passed through Cu turnings at 800 C & water scrubbed) caused a 90 % inhibition of the non-enzymatic formation of IAA and a 70 % inhibition of the enzymatic reaction with the mung bean preparation. This indicated an oxidative reaction with direct participation of O_2. Since the *o*-quinone is considered to be the product of catecholase action (30), a reaction requiring oxygen (ibid), the activity of *o*-benzoquinone was examined.

Benzoquinone was synthesized by the silver-oxide oxidation of catechol, using the method described by Tarpley (38). Freshly prepared ethereal solutions were shaken with 0.02 M aqueous solutions of tryptophan, pH 7.2. A reddish pigment (irreversibly decolorized by sulfuric acid) quickly appeared in the aqueous phase. After 1 hour, with occasional vigorous agitation, the acid fraction was separated from the aqueous layer and assayed for IAA. Nine μg were found.

INDOLEACETALDEHYDE: Tryptophan conversion to IAA in plant material is considered to go through either indolepyruvic acid or tryptamine to indoleacetaldehyde, the immediate precursor of the auxin (12, 29). Furthermore, oxidation of tryptophan by dicarbonyls such as isatin has been used as a source of indoleacetaldehyde (2, 27). The Strecker degradation of an α-amino acid by dicarbonyls (32, 34), a catalytic oxidation by quinones to the cyanohydrin, and the novel variant proposed by Spenser et al. (37) leading to an unstable carbinolamine, also suggest the intermediate formation of indoleacetaldehyde. The likelihood that the aldehyde participated in the polyphenol-mediated conversion of tryptophan to IAA was, therefore, explored.

The participation of tryptamine in the reactions between tryptophan and IAA would increase the probability of indoleacetaldehyde as an intermediate, inasmuch as the aldehyde is the first product of amine-oxidase action. Tryptamine · HCl, 50 mg, was incubated in the usual manner with catechol and catechol plus mung bean enzyme. No IAA was obtained from either medium. Thus, if tryptophan is decarboxylated to tryptamine by the catechol system, subsequent conversion of the amine to IAA apparently does not take place.

As a more direct approach, about 200 μg of indoleacetaldehyde was incubated with enzyme, with catechol, and with catechol plus enzyme. Though control flasks of aldehyde alone yielded no IAA, 6 μg of IAA were produced by adding just the enzyme. No auxin was found in the extracts of the mixtures containing catechol with or without enzyme. Evidently a component of the crude enzyme was able to oxidize the aldehyde to IAA, though the aldehyde is not so oxidized in the presence of the catechol system. Catechol without enzyme definitely did not convert indoleacetaldehyde to IAA, though the auxin is formed from tryptophan under similar conditions.

Further evidence that indoleacetaldehyde is not an intermediate in the phenol-mediated tryptophan to IAA reaction is provided by the effect of the aldehyde-reagent dimedon. The tryptophan-catechol reaction was carried out with the presence of 1.5 mg dimedon in a 10-ml volume. Control flasks without dimedon produced 11.2 mg IAA per hour, whereas those with dimedon yielded 12.6. Since dimedon is presumed to be specific for aldehydes (41) and reacts with indoleacetaldehyde (8, 17), a free aldehyde does not appear to be involved.

INDOLEPYRUVIC ACID: Oxidation of tryptophan to IAA via the keto-acid was then considered, since tryptamine and indoleacetaldehyde were evidently not intermediates. Indolepyruvate seemed particularly appropriate in view of its spontaneous breakdown in solution (4, 6, 17, 23) in part to IAA, an instability that is compatible with the formation of auxin by tryptophan and catechol without enzyme.

Following the procedure of Kaper and Veldstra (23), the acid fractions of tryptophan-catechol incubation mixtures (1 hr) were taken up in methanol and chromatographed in ascending isopropanol-ammonia-water 10:1:1. The developed strips were sprayed with a solution containing 5 % $HClO_4$-0.05 M $FeCl_3$ in a 50:1 ratio. Extracts of control incubations with synthetic indolepyruvate and of incubation blanks without tryptophan or catechol were also chromatographed. Five of the decomposition spots characteristic of indolepyruvic acid were found in the sprayed chromatograms of the extracts of the tryptophan-catechol reaction. They correspond to the spots A, D, IAA, H, and IAld described by Kaper and Veldstra. This indicates that indolepyruvic acid is formed during the phenolic degradation of tryptophan.

To evaluate this conclusion, we determined whether labeled indolepyruvate was formed when C^{14}-labeled tryptophan was incorporated into the medium. The incubation mixture consisted of 0.02 M D,L-tryptophan-3-C^{14} (New England Nuclear Corp.,

TABLE VII

FORMATION OF IAA BY ACTION OF THE PROLYL-CATECHOL COLOR COMPLEX UPON TRYPTOPHAN

CATECHOL	PROLINE	ENZYME	FERRICYANIDE	TRYPTOPHAN*	INCUBATION TIME, min	IAA, μg
+	+	−	−	−	60	0
+	+	+	−	−	60	0
+	+	−	+	−	15	0
+	+	−	+	−	60	0
+	+	−	+	+	30	5
+	+	−	+	+	60	8
+	+	+	−	+	30	8
+	+	+	−	+	60	12

* Tryptophan was added 15 minutes after the preceding components were combined. Phosphate buffer 0.05 M, pH 7.8, 30 C; catechol 0.005 M, proline 0.02 M, enzyme 42 mg, $K_3Fe(CN)_6$ 0.02 M, tryptophan 0.02 M.

5.82 mC/mM), catechol, and enzyme. After an incubation period of 1 hour, the acid and neutral fractions in ether were concentrated in vacuo and taken up in 95% ethanol. This was added directly to a 25 × 1 cm column of Solka-Floc cellulose powder which previously had been washed with dilute ammonium hydroxide, dilute hydrochloric acid, water, ethanol, and ether. The column was developed with a solvent mixture consisting of water-saturated butanol: 95% ethanol (9:1) to which was added 0.2 ml of concentrated HCl per 100 ml of solution. A parallel column was run with 10 mg of indolepyruvate; each 1 ml of eluate was tested with ammoniacal silver nitrate (Tollens' reagent) to indicate the position of the keto-acid. (Crystallization occurred in several eluates of the marker column that were refrigerated overnight.) The eluate from the column that had the incubation extract was reduced in vacuo to a volume of about 0.5 ml. Indolepyruvate, 20 mg, was added and the mixture was taken up in 95% ethanol. Water was slowly added until a cloudiness appeared and then 15 mg of *p*-nitrophenylhydrazine dissolved in glacial acetic acid was added. The mixture was refrigerated overnight to crystallize the *p*-nitrophenylhydrazone. This was successively recrystallized from hot alcohol three additional times. The specific activity of each batch of crystals, as measured by a windowless flow counter, was, respectively, 380, 280, 240, and 260 counts per minute per mg. These results corroborate the conclusion that indolepyruvate is formed during the phenol-mediated conversion of tryptophan to IAA.

ACTIVITY OF PROLYL-CATECHOL COLOR-COMPLEX: The formation of color complexes by the action of the catechol-oxidase system on amino acids has been investigated in some detail. Under suitable conditions, the complex formed spontaneously by catechol and an amino acid is identical with that formed enzymatically, and is able to deaminate a number of primary amino acids (3, 21, 22, 39).

Catechol was therefore incubated with a 4-mol excess of ferricyanide in the presence of proline at pH 7.8, conditions that rapidly oxidize the catechol (3, 22). A reddish-violet pigment formed quickly. As is indicated in table VII, no IAA could be detected either at 15 or 60 minutes, with or without enzyme, in the absence of tryptophan. (Similar results were observed with glycine in place of proline.) However, the color complex formed from proline and catechol by either ferricyanide or the enzyme converted subsequently added tryptophan to IAA. Of the two oxidation systems, the enzyme was more effective.

OPTIMUM REACTION CONDITIONS: The yields of IAA obtained with the catechol-tryptophan system by varying the incubation conditions are given in the succeeding tables and figure. In these experiments another lyophilized mung bean enzyme preparation of lower specific activity was used. It was prepared in a similar fashion (cf. Methods) from a cell-free supernatant fraction containing 36 mg of solids and 1.1 mg of TCA-precipitable nitrogen per ml. Except as noted, the customary incubation and assay procedures were used.

In table VIII it is indicated that a catechol concentration of 10^{-2} M yields relatively large amounts of IAA. The optimum concentration of catechol for the medium used is probably somewhat higher; however, the large amount of concomitantly formed oxidation products interfered with the assay for IAA.

As described under Methods, the pH of an incu-

TABLE VIII

EFFECT OF CATECHOL CONCENTRATION ON YIELD OF IAA

CATECHOL CONC. M	IAA μg/hr − ENZYME	IAA μg/hr + ENZYME
10^{-1}	tr.*	14*
10^{-1}	tr.*	19*
5×10^{-2}	12	18
5×10^{-2}	12	15
10^{-2}	10	15
10^{-2}	11	14
10^{-3}	3	6
10^{-3}	3	5
10^{-4}	0	0
10^{-5}	0	0

* Values unreliable because of excessive catechol oxidation products on chromatogram.

TABLE IX

EFFECT OF POST-INCUBATION pH ON APPARENT YIELDS OF IAA FROM CATECHOL & TRYPTOPHAN

pH*	IAA, μg	
	− ENZYME	+ ENZYME
7.5	tr.	13.3
8.0	tr.	15.1
9.2	7.5	17.8

*pH During incubation was 7.2.

bation mixture was adjusted to 9.2 after enzyme inactivation for removing basic and neutral fractions. We found that this pH adjustment contributed to the yield of IAA from the trytophan-catechol reaction, both with and without the enzyme present. This effect, illustrated by table IX, is probably caused by an increase in the spontaneous oxidation rate of catechol as the hydrogen ion concentration decreases (3). The table indicates that the contribution of the enzyme over the spontaneous rate could be enhanced at pH < 9. This enhancement of the enzyme contribution is also suggested by the curves in figure 1, which show the effect of varying the pH of the incubation medium.

Figure 1 indicates that the spontaneous oxidation yield of IAA is maximal at ~ pH 9.5, whereas the optimum for the enzymatic system is ~ 8.5. Without the enzyme, no IAA was found under the conditions of pH 5 to 7. Since the most alkaline solutions darkened quite rapidly, the drop in yields therein might be attributed to competitive humin formation. The amount of IAA formed from tryptophan by alkaline decomposition (18) would be insignificant relative to the amounts of IAA dealt with here.

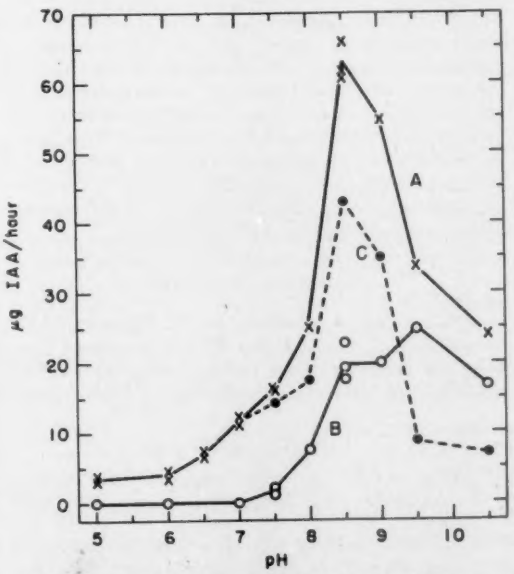

FIG. 1. The influence of pH of the incubation medium on the yield of IAA. All components preadjusted to indicated pH with HCl or NaOH, and similarly adjusted to pH 8.2 before the fractionation sequence. A, with enzyme; B, without enzyme; C, A minus B.

DISCUSSION

The action of a phenol-phenolase system upon tryptophan is a new reaction sequence for the production of auxin (31). Its characteristics are consistent with currently proposed mechanisms for the oxidative deamination of amino acids by the phenolase complex (cf. 9, 30, 39). These mechanisms hold that oxidation of a vicinal dihydroxy phenol to the corresponding *o*-quinone is catalyzed by phenolase. The quinone condenses spontaneously with amino acids to form a *p*-substituted reddish pigment of the aminoquinone class. This pigment reacts again with free amino acid to form the *o*-quinonimine as a second condensation product. The quinonimine spontaneously rearranges, hydrolyzes, and is reoxidized, presumably to the primary pigment; in this process, ammonia and a carbonyl compound are liberated.

The observations in the present study may be considered in terms of the above framework.

A. We have shown that enzyme preparations from the mung bean seedlings, as well as the oat and sunflower, form comparatively large amounts of auxin from tryptophan in the presence of catechol.

B. Other *o*-dihydroxy phenols, or compounds readily converted to that structure, can replace catechol, with similar orders of activity. The nature of the *p*-substituent appears to be relatively nonspecific.

C. The catechol system is inhibited by reducing and chelating agents; the copper-reagent diethyldithiocarbamate is particularly effective.

D. Mushroom tyrosinase can substitute for the mung bean enzyme in the tryptophan-catechol system; a polyphenolase action of the mung enzyme may be inferred. Using auxin formation as a criterion, the pH optimum with the mung bean enzyme is 8.5.

E. A polyphenolase action of the enzyme would use O_2 as an acceptor and form a quinone as the first product. We find that the action of the mung bean enzyme-catechol system is inhibited in a N_2 atmosphere and that the formation of IAA from tryptophan can also be accomplished by contact of the amino acid with ethereal solutions of *o*-benzoquinone.

F. An analogous model can be constituted with other amino acids that are inactive per se in producing auxin. For example, catechol reacts with proline in the presence of ferricyanide as an oxidant. The complex so produced forms IAA upon the subsequent addition of tryptophan.

G. Our data indicate that indolepyruvic acid is formed by the reaction of phenols with tryptophan, and that indolepyruvate, not tryptamine or indoleacetaldehyde, is the intermediate in the phenol-medi-

ated conversion of tryptophan to IAA. This conclusion is compatible with the formation of a carbonyl product on breakdown of the quinonimine-amino acid complex. The non-enzymatic formation of IAA here observed is also compatible with the decomposition characteristics of indolepyruvate, which degrades spontaneously to IAA.

We believe that the experiments described permit the conclusion that tryptophan is oxidatively deaminated (*22*) to indolepyruvate by oxidized dihydric phenols; the keto-acid then breaks down spontaneously to IAA. The chief action of the mung bean enzyme is that of a phenolase, i.e., catalysis of the primary oxidation of phenols to the *o*-quinoid form, though the enzyme may also channel or enhance the conversion of indolepyruvate to IAA (cf. 15).

The auxin produced in these phenolic reactions with tryptophan has not been rigorously shown to be IAA. We have assumed that it is IAA because the substance: A: behaves as an organic acid in aqueous ether solvent systems; B: is an auxin by both Avena section and curvature tests; C: forms a chromophore with the iron-perchloric acid reagent that has the same absorption spectrum as the chromophore formed by IAA; D: has the same migration rate as IAA on chromatography with *n*-butanol: ammonia: water, 10:1:1, (R_f 0.35) and acetic acid (0.1 N): methanol: *n*-butanol, 98:1:1 (R_f 0.60), using both colorimetric and biological criteria of distribution on the chromatogram.

Does the interaction between phenols and tryptophan represent the usual pathway of auxin biogenesis? The distribution of biological activity on chromatograms of the acid fraction from the reaction of tryptophan and the mung bean enzyme is qualitatively similar to the distribution obtained from the reaction of tryptophan, catechol, and enzyme. However, we feel that the above pathway may not be followed in *normal* tissue on the basis of the following two considerations.

First, there are indications that the phenolases of plants are associated with cellular particulates (*7*). Yet none of the essentially intact cell organelles produce IAA from tryptophan in the absence of catechol (*14*); such activity is a soluble component of the cytoplasm. Though the preceding can hardly be considered convincing evidence, the phenols and phenol oxidase in intact cells are very probably spatially separated. The tryptophan-phenolase-phenol reaction could thus be considered as a latent, potentially operative, mechanism for auxin formation in the plant. Its components normally would be compartmentalized in vivo and so prevented from interaction at significant rates. The lysis which occurs after wounding of plant tissue, wherein activation of the phenolase complex takes place, may then produce abnormal quantities of growth hormone via the phenol-tryptophan reaction. Callus and gall formation could be histogenic consequences of such hyperauxiny, and there would be no need to postulate the function of a specific wound hormone such as traumatin in the morphogenesis of wound regeneration.

Second, normal auxin biogenesis is highly sensitive in vivo to X- and gamma-radiation (*13, 35*). This radiosensitivity is rapidly manifest in three ways: as a reduced level of the free hormone, as alterations in morphological phenomena dependent upon hormonal concentration, and as a reduction in activity of the soluble enzyme converting tryptophan to auxin. In current experiments we have found that the phenolase-mediated conversion of tryptophan to IAA is unimpaired in preparations obtained from seedlings immediately after exposure to single X-ray doses of 5 kr.

Since the phenolase complex probably is activated by the disruptions in cellular integrity accompanying chronic high-energy irradiation, a consequent hyperauxiny could well be the basis for the hyperplasia and neoplasms that are commonly observed (*36*) in such irradiated plants.

Summary

Phenols, under conditions leading to their oxidation, react with tryptophan to form the auxin indoleacetic acid. The action is catalyzed by polyphenolase enzymes of mung bean, oat, and sunflower, and occurs spontaneously at alkaline pH. Various *ortho*-dihydric phenols, or phenols readily oxidized to that form, are effective. The reaction is inhibited by replacement of air with N_2, and by reducing and chelating agents. It can be duplicated by mushroom tyrosinase, by exposure of tryptophan to ethereal solutions of *o*-benzoquinone, or by adding tryptophan to a prolyl-catechol pigment formed via either ferricyanide or enzymatic oxidation. It is shown experimentally that indolepyruvic acid and not tryptamine or indoleacetaldehyde is the probable intermediate in the conversion of tryptophan to indoleacetic acid by the enzyme system. These observations are consistent with current concepts of oxidative deamination of amino acids by quinonimines. The pertinence of this new pathway of auxin biosynthesis is discussed in regard to normal auxin biogenesis, wound regeneration, and radiation-induced hyperplasia.

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American Society of Plant Physiologists, *Executive Committee Meeting, 27 August 1961, Purdue University, Lafayette, Ind.* The Executive Committee meeting was called to order at 8:40 PM by President Aubrey Naylor. Those present were: S. Aronoff, H. Beevers, A. H. Brown, R. H. Burris, S. B. Hendricks, W. H. Klein, P. J. Kramer, R. W. Krauss, B. S. Meyer, C. O. Miller, A. W. Naylor, L. M. Rohrbaugh, F. Skoog, B. Stowe, H. J. Teas, and F. Went.

The minutes of the previous meeting were approved as printed in PLANT PHYSIOLOGY.

The president gave his report and outlined the possibility that a five-year grant from the Charles F. Kettering Foundation will be established to make an annual award for work in photosynthesis. A motion was passed instructing the president to accept such a grant and to appoint a committee to administer it. President Naylor also reported that the Kettering Foundation had awarded the society a grant of $700 to bring Dr. Duysens to this country for the meetings.

The executive secretary-treasurer, W. H. Klein, presented his annual report. During the year the membership climbed to 1,723, and the net worth increased from $87,217.98 to $89,028.65. The net operating loss for the fiscal year 1960 to 1961 was almost $3,300. Dr. Klein's budget of $3,500 for the coming year was approved; he was instructed to go ahead with the preparation and publication of a new directory, the cost of which may run close to $2,000. His report was accepted; some of its details follow:

Membership Totals

	June 30, 1961	Increase over 1959-60
Life members	38	3
Foreign members, regular	246	31
Foreign members, students	10	8
U.S. members, regular	1,204	29
U.S. members, student	192	14
Corresponding members (excluding full members)	13	
Emeritus members	20	3
Totals	1,723	88

Subscriptions

U.S. subscriptions	V. 35	517	V. 36	530
Foreign subscriptions	V. 35	977	V. 36	1,105
Complimentary U.S.	V. 35	5	V. 36	7
Total Increase		143		

Summary of Cash Accounts

Cash on hand, June 30, 1960	
Checking account	$18,895.20
Reserve accounts	16,535.28
Total	$35,430.48
Receipts 1960–61	41,592.04
Total	$77,022.52
Expenditures 1960–61	39,894.73
Balance	$37,127.79
Cash on hand, June 30, 1961	
Checking account	$17,615.59
Reserve accounts	19,512.20
Total	$37,127.79

Dr. A. H. Brown, editor of PLANT PHYSIOLOGY, reported on the activities of his office during the past year. Upon the recommendation of the Editorial Board, Drs. George Laties and Emanuel Epstein were appointed to the board for terms of five years each. The editor's 1961 to 1962 budget of $40,695 was approved as was his report. Since the editor's term ends in August 1962, President Naylor was authorized to consult with Editor Brown and to appoint a small committee to be selected from this Editorial Board which will be charged with choosing a new editor. Dr. Brown indicated that he prefers not to serve on this committee, but is willing to act in an advisory capacity if asked to do so. The substance of Dr. Brown's report follows:

The editor's annual report is supposed to reveal something about the condition of our Journal. To this end the editor supplies tabulations showing how manuscripts have been handled—i.e., how much we are publishing and how long it takes us to publish it. He also provides a financial accounting to show what it all costs. For at least some of us statistics have an ill-defined fascination and all of us are interested in budgets when it is our own money that is involved. Nevertheless, these items which make up the bulk of the editor's report fall well short of telling about the true condition of our Journal. That can be judged only by our readers' opinions of it, by the quality—both scientific and literary—of the articles we publish, by the readiness with which contributors of good papers send their manuscripts to PLANT PHYSIOLOGY rather than to other journals. To assess the

condition of our Journal in this sense an editor's report cannot be of much help. The proof of our pudding is in the eating and, we may add, when the taste is in some way not to your liking, you are invited to complain to the cook.

MANUSCRIPT PROCESSING. The following tabulation includes manuscripts received between 11 August 1960 and 10 August 1961 as well as hold-overs which were on hand at some stage of processing at the time of last year's tabulation. Discrepancies among subtotals are due to some manuscripts appearing in more than one category.

New manuscripts received		185
Accepted for publication		144
With little or no revision	90	
After major revision	54	
Rejected		54
Withdrawn		11
Papers now in process		59
Awaiting assignment to reviewers	10	
In hands of reviewers	17	
Awaiting editor's decision	7	
Awaiting return by author	25	
Papers in press		43
September issue	33	
November issue	10	

In the above tabulation the categories, "rejected" and "withdrawn", deserve special explanation. The total number of manuscripts reported for these items is substantially larger than in any other year since 1954. We hasten to assure you that this is neither reflection on the average quality of contributed manuscripts nor evidence of tightening standards. We have simply used more rigorous criteria this year for deciding whether to classify unacceptable manuscripts as actually "rejected" or as "awaiting revision by author". In many instances nothing further is heard about such manuscripts. They move from the active to the inactive file. Finally they are forgotten. They never can be properly counted in our totals unless, after the passage of a reasonable number of months, they are frankly acknowledged as withdrawn or rejected, whichever seems more appropriate.

Had we employed precisely the same criteria as formerly this year's rejection percentage would not have been significantly higher than usual. We believe our current system of classifying manuscripts is an honest one and we shall continue to employ it in the future.

Comparison with counts from previous years indicates that the number of manuscripts on hand (an inverse measure of our processing efficiency) has been steadily decreasing as the following table shows:

Year	Manuscripts in process
1960–61	59
1959–60	72
1958–59	91
1957–58	89

The trend reflects improvements in our own procedures and in fitting our schedules to those of our printer. The credit for it goes not to the editor but rather to his assistants and to Mr. Wylie, the plant superintendent at Craftsmen, Inc.

TIME STUDY ON MANUSCRIPTS. Last year we reported that the average time from receipt of a manuscript until its appearance as a printed article was 9.2 months. We were not especially proud of that figure for we were sure we could do better. This time the record shows improvement; the overall time has been reduced to 7.2 months. Possibilities for further reduction in time have not yet been exhausted. Below is a breakdown of the manuscript processing schedule showing how the time was spent along the way:

Time from receipt of manuscript until:	
Arrival of 2nd review	7.9 wk
Editor's letter of decision	9.8
1st return of revised manuscript by author	15.6
Publication	31.2

During the past year all Journal issues have appeared in the same month as the date of issue. We confidently expect this practice to continue. Our arrangements with Craftsmen, Inc., include a workable countdown for the several stages preceding actual publication. Thus we (& the printer) are able to plan on, and to meet, a succession of deadlines which —emergencies aside—should continue to insure publication of each issue on time.

COSTS. The Journal budget has two major categories: expenses of the editorial office and charges for printing and mailing. Each year we have estimated our editorial office expenses fairly accurately. Not so the printing and mailing costs. This has been due largely to our inability to predict the number of pages to be published in each forthcoming year; our printing costs depend closely on the number of pages published.

The editorial office expenses have increased gradually over the past few years—partly because of increased work volume and partly because of salary level increases. The following comparison shows the upward trend:

	1957-1958	1958-1959	1959-1960	1960-1961
Editorial assistant	$2,480	$2,902	$1,875	$2,095
Clerk-typist	...	...	986	1,043
Editor's stipend	1,000	1,000	1,000	1,000
Postage	378	254	503	540
Office supplies	82	56	104	88
Telephone & telegraph	11	62	47	61
TOTAL	$3,951	$4,275	$4,515	$4,827

Of our total costs, about 15 % are those of the editor's office, 4 % are for journal mailing, and about 80 % for printing.

Our printing bill this past year was well above expectations and in view of our conservative prediction of a year ago we ran a substantial deficit over authorized expenses.

The costs for printing and mailing the last six issues of our Journal were almost one-third more than for the preceding six issues. Part of this increase is attributable to the printing of 500 more copies of the Journal (20,600 in 1959–60 vs. 21,100 in 1960–61). Part of it was due to increased mailing costs. However, most of the increase was accounted for by the fact that we printed more pages (760 in 1959–60 vs. 1,000 in 1960–61).

It may be instructive to look at several aspects of our costs. Considering first only printing and mailing, our cost per page has not changed significantly over the past year. It was $0.84 per 100 copies in 1959 to 1960 and $0.82 per 100 copies in 1960 to 1961. Nevertheless, larger issues mean more paper and a higher printing and mailing cost per issue. Thus our printing, mailing, and editorial office cost *per copy* rose from $1.29 in 1959 to 1960 to $1.60 in 1960 to 1961. As nearly as we can estimate, then, each issue of our Journal cost $1.60 or $9.60 for six issues, a number which may be of at least incidental interest when compared with our membership fee.

In last year's annual report we pointed out what perhaps is a trend toward publication of more pages per volume. The uncertainties of extrapolation prevented us from calling the trend alarming but the implication was clear. Increased pages mean increased costs. If we receive a greater number of manuscripts (or, on average, longer ones) either we become more choosy or we must pay more to publish a larger Journal. Last year's volume (35) is the largest we have published since we adopted the present format in 1954. Volume 36 will be about as large if not larger than volume 35.

Our editorial policy has been to publish all manuscripts which come up to the standards we are trying to maintain. No manuscript has been rejected for lack of space. No article has been delayed waiting for publication space to become available. While the editor has insisted on condensation of many manuscripts and on deletion of parts of manuscripts, in every case this was in the interests of manuscript improvement; never has he insisted on shortening an article merely to save space. If this policy is continued, it seems likely that our Journal volumes will continue to increase in size and in overall cost. On the assumption that our present policy is a desirable one, the budget shown below is proposed for 1961 to 1962.

This budget takes into account an increase in number of pages to be published (estimated at 20 %). It provides for salary increases for the editor's staff (circa 6½ %). It anticipates an increase in postage rates (25 % on first class mail & ¼c per copy for mailing the Journal). These extra mailing costs should amount to $200 per year. The major item, printing, and mailing was estimated as the product of our current cost per page ($0.82 per 100 copies), pages per year (1,200), and copies per issue (3,600). The least reliable figure is, of course, the total number of pages.

PROPOSED BUDGET, 1961–62

Editorial office	
Editorial assistant	$ 2,254
Clerk-typist	1,123
Editor's stipend	1,000
Postage	675
Office supplies	100
Telephone & telegraph	65
	$ 5,217
Printing & mailing	35,478
TOTAL	$40,695

—ALLAN H. BROWN, *Editor-in-Chief*

The secretary reported the nomination and election of new officers. The counting of ballots was carried out by the secretary, David Blaydes, and William Hopkins. The following were elected:

President—Harry Beevers
Vice-president—Carlos Miller
Secretary—Sam Aronoff
Member of Executive Committee—
Sterling Hendricks
Members of Editorial Board—P. J. Kramer
K. V. Thimann

Dr. Naylor next gave a report concerning the recommendations of a group interested in forming a federation of plant scientists. After much discussion of this subject, two motions reached a vote. The first motion was voted down. It stated that "The Executive Committee of the American Society of Plant Physiologists favors setting up within the AIBS a plant science group." The second motion carried. It stated that "The Executive Committee of the American Society of Plant Physiologists favors strengthening the AIBS so as to do a better job of representing all biologists."

Dr. B. S. Meyer reported the activities of the AIBS and its governing board. He urged more cooperation on the part of our Society in terms of comments and suggestions from officers and other members.

Reports followed from R. W. Krauss for the National Research Council, J. van Overbeek for the American Standards Association, M. A. Sprague for the Joint Committee on Grassland Farming, and F. Went for the International Association of Plant Physiologists. President Naylor was instructed to make known by letter that our Society favors having the physiology program for the 1964 International Botanical Congress organized in consultation with the International Association of Plant Physiologists.

Dr. G. Ray Noggle, appointed to the Board of Trustees, is to serve a three-year term beginning 1 January 1962. Dr. H. J. Dyer, appointed to the

Council of the AAAS, is to serve until 1 January 1962; Dr. L. M. Rohrbaugh is to serve with the same group until 1 January 1963.

After considerable discussion the Executive Committee decided to add to the general fund the $2,500 donated to our society by the late Dr. Walter Thomas, and to maintain no identity of the money; the name of Walter Thomas, however, will be kept on a permanent list of major contributors.

Several other items were mentioned without any action; the committee adjourned about 30 minutes after midnight.

—C. O. MILLER, *Secretary*

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Business Meeting, 29 August 1961, Purdue University, Lafayette, Ind.* President A. W. Naylor called the meeting to order at 4:15 PM. There were approximately 75 members present.

The minutes of the previous annual meeting were approved. The minutes of the Executive Committee meeting were summarized and accepted.

Dr. Naylor outlined the proposed award in photosynthesis from the Charles F. Kettering Foundation. A motion was passed that the Society should accept the grant and that the president should appoint a committee to carry on the awards program. A resolution also was passed thanking the Kettering Foundation for its help in bringing Dr. Duysens to this country for the meetings.

Considerable discussion occurred about the proposed federation of plant scientists. The group present passed a motion in support of the two motions previously passed by the Executive Committee on this subject. Also, another motion carried; it is to the effect that the Society stands ready to increase assessments so as to strengthen the AIBS.

The executive secretary-treasurer, Dr. W. H. Klein, gave a brief report; this was followed by a report from the editor of Plant Physiology, Dr. A. H. Brown. Both of these reports emphasized the fact that the Society is currently undercharging for its services. To correct this situation, a motion was passed; it states that "Dues and subscription fees should be increased, the extent being determined by the Executive Committee, and the effective date being 1 January 1962."

Dr. Sam Aronoff expressed thanks to Dr. Harry Beevers for his excellent work in serving as our local representative for the Purdue meetings and to Dr. Alfred Sussman, who was a member of the Program Committee.

President Naylor adjourned the meeting at about 5:20 P.M.

—C. O. MILLER, *Secretary*

CHARLES REID BARNES AWARD. Dr. Frank P. Cullinan, associate director of the Agricultural Research Service, U.S. Department of Agriculture, was elected to a Charles Reid Barnes Life Membership in the American Society of Plant Physiologists. Dr. Cullinan was born in Geneva, N.Y.; he graduated from Cornell University in 1917, and did his doctorate at the University of Chicago. He was at Purdue University from 1917 to 1931, and has been with the U.S. Department of Agriculture since then. He was secretary-treasurer of the Society from 1937 to 1938 and president in 1940. Dr. Cullinan has served without stint on large and small jobs for plant physiology and the Society. He was chairman of the AIBS during its early period, is currently secretary-treasurer, and was president of the American Society of Horticultural Scientists in 1953.

—W. E. LOOMIS, *Chairman*,
Award Committee

TREE PHYSIOLOGY WORKSHOP MEETS AT CLEMSON COLLEGE. The first Southern Forest Tree Physiology Workshop was held Sept. 6, 7, and 8, 1961, at Clemson College, Clemson, S. C. Twenty-eight invited researchers met to become acquainted with one another's work and to form personal contacts conducive to free exchange of ideas and information among those interested in similar phases of tree physiology research. All participants are actively engaged in tree physiology research from Maryland to Texas.

There were no formal papers presented at the workshop. Each session was a round-table discussion on a general topic led by a moderator, everyone being free to enter into the discussion. The session topics and moderators were: Growth and development I. Internal factors—R. M. Allen. Growth and development II. Environmental effects—R. C. Hare. Absorption, translocation, and elimination of water, solutes, and gases—Holger Brix. General plant metabolism and biochemistry—Robert L. Barnes. General session—Business session—Mason C. Carter.

The workshop was conceived and called by an informal committee composed of R. M. Allen, U.S. Forest Service; Robert L. Barnes, U.S. Forest Service; Mason C. Carter, Auburn University; and Wm. H. Davis McGregor, Clemson College. The Department of Forestry at Clemson College was host-sponsor of the conference. The group voted to hold a similar meeting in 1963.

GENERAL INDEX

—A—

ABELES, FREDRICK B. Stimulation of Hill reaction by carbon dioxide, 202

Absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity. I. Penetration & translocation of Cl^{36}- and C^{14}-labeled dalapon, 688

Absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity. II. Distribution & metabolic fate of dalapon in plants, 698

Absorption of phosphorus applied to leaves of sunflower, effect of soil moisture stress on, 762

Absorption, rubidium, kinetics of, by barley, 183

Accumulation & transformation of sugars in stalks of sugar cane. Origin of glucose & fructose in the inner space, 175

Acetate, labeled, 2,4-dichlorophenoxyacetic acid effect on utilization of, by bean leaf & stem tissues, 192

Action spectra of light-saturated photosynthesis, 114

ADAMS, DONALD F. Variations in starch & total polysaccharide content of *Pinus ponderosa* needles with fluoride fumigation, 261

Adenylate kinase involvement in phosphorylation of ribose-5-phosphate by adenosinediphosphate in mitochondria-free preparations from rough lemon leaves, 368

Agrobacterium bacterium, DNA content of broad bean internodes in connection with tumor induction by, 216

AKAZAWA, T. Analytical study of ipomeamarone & chlorogenic acid alterations in sweet potato roots infected by *Ceratocystis fimbriata*, 139

ALBERT, LUKE S. Effect of boron on elongation of tomato root tips, 244

Algae, mass culture of, 342

ALSTON, RALPH E. See ARNOLD, ALLAN, 650

Alyssum saxatile, salt tolerance & protoplasmic salt hardiness of, 478

AIBS Style manual, 715

American Society of Plant Physiologists, business meeting, 1960, 124

American Society of Plant Physiologists, business meeting, 1961, 850

American Society of Plant Physiologists, executive committee meeting, 1960, 121

American Society of Plant Physiologists, executive committee meeting, 1961, 847

American Society of Plant Physiologists, midwestern section, 715

American Society of Plant Physiologists, northeastern section, 520

American Society of Plant Physiologists, southern section, 359

Amino acid treatment, production of yellow strapleaf of chrysanthemum & similar disorders by, 197

Ammonia assimilation by nitrogen-starved cells of *Chlorella vulgaris*, 208

Anabaena cylindrica, pteridines in, 240

Anabaena variabilis, pteridines in, 240

Anacystis nidulans, pteridines in, 240

Analytical study of ipomeamarone & chlorogenic acid alterations in sweet potato roots infected by *Ceratocystis fimbriata*, 139

ANDREAE, W. A. Studies on 3-indoleacetic acid metabolism. VII. Metabolism of radioactive 3-indoleacetic acid by pea roots, 783

Anthocyanin production in detached petals of *Impatiens balsamina* L., 1

Antirrhinum majus, salt tolerance & protoplasmic salt hardiness of, 478

ARNOLD, ALLAN. Certain properties of hypocotyl of *Impatiens balsamina* reflecting physiological complexity, 650

Ascorbic acid, photooxidation of, 252

ASOMANING, EDWIN J. A. Comparative study of phototropic response & pigment content in oat & barley coleoptiles, 453

Aspergillus niger, isolation & characterization of malformin, 30

Aspergillus niger, studies on response of bean seedlings & corn roots to malformin, 37

Assimilation of ammonia by nitrogen-starved cells of *Chlorella vulgaris*, 208

Assimilation of C^{14}, N^{15} labeled urea by excised apple & peach leaves, 757

Auxin-boron relationship in C^{14} translocation in bean plants, 672

Auxin concentrations, high, steady state growth of Avena coleoptile sections in, 323

Auxin formation from tryptophan through action of polyphenols, 838

Auxin, mediation of geotropic response by lateral transport of, 364

Auxin-phenol complexes, 589

Avena sativa, comparative study of phototropic response & pigment content in coleoptiles, 453
Avena sativa, growth & geotropic curvature of coleoptiles in presence of *n*-1-naphthylphthalamic acid, 727
Avena sativa, steady state growth of coleoptile sections in high auxin concentrations, 323
AWARD, CHAS. REID BARNES, 850
AXELROD, BERNARD. See EVANS, WILLIAM R., 9

— B —

BACH, MICHAEL K. Correlation between inactivation of 2,4-dichlorophenoxyacetic acid & cessation of callus growth in bean stem sections, 89
BACH, MICHAEL K. Mechanism of stimulation of respiration by purines: I. Inhibitor studies, 317
BACH, MICHAEL K. Metabolites of 2,4-dichlorophenoxyacetic acid from bean stems, 558
BACH, MICHAEL K. Ubiquity of stimulation of endogenous respiration by purines & purine analogs, 85
BACON, JAMES A. See OSBORNE, THOMAS S., 309
BAKER, JAMES E. Assimilation of ammonia by nitrogen-starved cells of *Chlorella vulgaris*, 208
BANDURSKI, R. S. See HAMILTON, R. H., 354
BANDURSKI, R. S. See KIVILAAN, A., 605
BARNES, CHAS. REID, Award, 850
Basis of shoot response to root temperature in tomato, 153
BASLER, EDDIE. Effects of moisture stress on absorption, translocation, & distribution of 2,4-dichlorophenoxyacetic acid in bean plants, 573
BEAMAN, T. CABRERA. See KIVILAAN, A., 605
BEAN, ROSS C. See TODD, GLENN W., 69
BEEVERS, HARRY. See BENEDICT, C. R., 540
BELKENGREN, R. O. Western X-little cherry virus effects on respiration of host tree, 824
BENEDICT, C. R. Formation of sucrose from malate in germinating castor beans. I. Conversion of malate to phosphoenol-pyruvate, 540
BENSON, N. R. Translocation & re-use of boron in broccoli, 296
Berberis thunbergii, salt tolerance & protoplasmic salt hardiness of, 478
BERRY, WADE L. See ULRICH, ALBERT, 626
BERTHOLD, ROBERT V. See CROSBY, DONALD G., 48
Betula alleghaniensis, dormancy as a quantitative state, 643
Betula lutea, dormancy as a quantitative state, 643
Betula pubescens, dormancy as a quantitative state, 643
BIDDULPH, O. Transpiration stream & ascension of calcium, 429
Blue green algae, pteridines in, 240
BONNER, JAMES. See KEFFORD, N. P., 323
Boron-auxin relationship in C^{14} translocation in bean plants, 672
Boron deficiency in tissue culture, cell wall carbohydrates in tobacco pith parenchyma as affected by, 336
Boron distribution in leaves, 420
Boron effect on elongation of tomato root tips, 244
Boron translocation & re-use in broccoli, 296
BORTHWICK, H. A. See TOOLE, VIVIAN K., 285
Botrydiopsis alpina, action spectra of light-saturated photosynthesis, 114
Brassica caulorapa, stimulation of Hill reaction by carbon dioxide, 202
Brassica napus, pyrimidine metabolism in germinating seedlings, 9
Brassica oleracea, translocation & re-use of boron in, 296
BRAY, J. R. Estimate of a minimum quantum yield of photosynthesis based on ecologic data, 371
BRIGGS, WINSLOW R. See GILLESPIE, BARBARA, 364
BROWN, A. H. See ABELES, FREDRICK B., 202
BROWN, J. C. See TIFFIN, LEE O., 710
BRUN, WM. A. Photosynthesis & transpiration from upper & lower surfaces of intact banana leaves, 399
BRUN, WM. A. Photosynthesis & transpiration of banana leaves as affected by severing vascular system, 577
BRYAN, A. M. See KUPILA, SIRKKA, 212
BURLEY, J. WM. A. Carbohydrate translocation in raspberry & soybean, 820
BURRIS, R. H. See TAMAOKI, TAIKI, 347
Business meeting, American Society of Plant Physiologists, 1960, 124
Business meeting, American Society of Plant Physiologists, 1961, 850
BUTTS, JOS. S. See FANG, S. C., 192

— C —

Calcium effects on oxalic, malic, & citric acid content of Valencia orange leaf tissue, 99
Calcium, essential role of in selective cation transport by plant cells, 437
Calcium in excised barley roots, uptake of magnesium & its interaction with, 290
Calcium influence on selectivity of ion absorption process, 58
Calcium, transpiration stream & ascension of, 429
Calcium uptake by excised barley roots, 53
Calcium uptake in roots of *Zea mays*, 766
Callus growth in bean stem sections, 89
Carbohydrate translocation in raspberry & soybean, 820
Carbohydrate, cell wall, in tobacco pith parenchyma as affected by boron deficiency & by growth in tissue culture, 336
Carbon dioxide fixation by auxin treated tissue, 374
CO_2 Fixation, in vitro, in sweet orange leaves, cyanide & chelating agent effects on, 118
Carbon dioxide, stimulation of Hill reaction by, 202
CARLTON, BRUCE C. Effects of ethylene & oxygen on production of a bitter compound by carrot roots, 550

CARRIER, WM. L. See HABER, ALAN H., 351
CASTLE, JOHN E. See MUMFORD, FRANKLIN E., 752
CATHEY, H. M. See TOOLE, VIVIAN K., 663
Cation effect on germination & germ tube development of *Puccinia coronata* uredospores, 14
Cation effect upon absorption of carrier-free cesium, 66
Cation transport, selective, by plant cells, essential role of calcium in, 437
Cell wall carbohydrates in tobacco pith parenchyma as affected by boron deficiency & by growth in tissue culture, 336
Cell wall preparations from corn coleoptiles, enzymatic activities associated with, 605
Celosia argentea, salt tolerance & protoplasmic salt hardiness of, 478
Ceratocystis fimbriata, changes in nitrogen metabolism in sweet potato with, 770
Certain properties of hypocotyl of *Impatiens balsamina* reflecting physiological complexity, 650
Cesium absorption, effect of cations upon, 66
Changes in nitrogen metabolism in sweet potato with black rot, 770
Changing respiratory pathways in potato tuber slices, 20
Chenopodium album, foliar penetration by chemicals, 169
Chenopodium amaranticolor, translocated heat injury, 721
CHERRY, JOE H. Effects of X irradiation on corn seed, 566
CHERRY, JOE H. Nucleotide & ribonucleic acid metabolism of corn seedlings, 163
Chlorella ellipsoidea, mass culture of algae, 342
Chlorella pyrenoidosa, action spectra of light-saturated photosynthesis, 114
Chlorella pyrenoidosa, reductive pentose phosphate cycle, 409
Chlorella pyrenoidosa, time course of oxygen evolution during photosynthesis in synchronized cultures of, 232
Chlorella pyrenoidosa, valine biosynthesis, 598
Chlorella vulgaris, assimilation of ammonia by nitrogen-starved cells of, 208
Chlorella vulgaris, mass adaptation to selenomethionine in populations of, 502
Chlorella vulgaris, mechanism of stimulation of respiration by purines, 317
Chlorella vulgaris, stability & reversibility of adaptation to selenomethionine in, 509
Chlorine uptake in roots of *Zea mays*, 766
Chlorogenic acid alterations in sweet potato roots infected by *Ceratocystis fimbriata*, 139
Chlorophyll synthesis & red, far-red response, 733
Chloroplast preparations, light dependent oxygen metabolism of, 252
Chloroplasts distinguished by use of labeled oxygen, cyclic & non-cyclic photophosphorylation in, 44
Chlorosis, fluoride induced, mechanism(s) of, 385
Chlorosis in higher plants as related to organic acid content, 415
CHMELIR, I. C. See BENSON, N. R., 296
Chrysanthemum morifolium, production of yellow strapleaf & similar disorders by amino acid treatment, 197
Citric acid content of Valencia orange leaf tissue, effects of calcium, potassium, & magnesium on, 99
Citrullus vulgaris, assimilation of C^{14}, N^{15} labeled urea by excised leaves, 757
Citrus aurantium, effect of soil oxygen deficit on iron nutrition of seedlings, 425
Citrus jambhiri, involvement of adenylate kinase in phosphorylation of ribose-5-phosphate by adenosinediphosphate in mitochondria-free preparations from rough leaves, 368
Citrus limon, photosynthesis & respiration in developing fruits, 69
Citrus sinensis, cyanide & chelating agent effects on in vitro CO_2 fixation in leaves, 118
Citrus sinensis, effect of sodium bicarbonate on iron absorption by, in seedlings, 52
Citrus sinensis, photosynthesis & respiration in developing fruits, 69
CLAUSS, HEINZ. See NELSON, C. D., 581
Cobalt influence on nitrogen fixation by Medicago, 73
Coconut milk, hexitols in: their role in nurture of dividing cells, 492
Cocos nucifera, hexitols in, their role in nurture of dividing cells, 492
COLLINS, FLOYD I. See CHERRY, JOE H., 566
Comparative study of phototropic response & pigment content in oat & barley coleoptiles, 453
CONRAD, HERBERT M. Interaction of gibberellic acid & allyl trimethylammonium bromide upon growth of Ulothrix, 685
Conversion of nicotine to nornicotine in harvested tobacco: Fate of methyl group, 592
Corn kernels, isolation of indole-3-acetic acid from, 354
Correlation between inactivation of 2,4-dichlorophenoxyacetic acid & cessation of callus growth in bean stem sections, 89
CORY, R. See BIDDULPH, O., 429
COUEY, H. MELVIN. Effect of cations on germination & germ tube development of *Puccina coronata* uredospores, 14
Critical phosphorus levels for lima bean growth, 626
CROSBY, DONALD G. Naturally occurring growth substances. II. Improved straight growth test & its applications, 48
Crown-gall tissue cultures of tomato, oxidation of reduced diphosphopyridine nucleotide by mitochondria from, 347
Cucumus sativus, photosynthesis & respiration in developing fruits, 69
Cucumis sativus, translocated heat injury, 721
CURRIER, H. B. See DYBING, C. DEAN, 169
CURTIS, ROY W. See TAKAHASHI, NOBUTAKA, 30

CURTIS, ROY W. Studies on response of bean seedlings & corn roots to maliformin, 37
Cyanide & chelating agent effects on in vitro CO_2 fixation in sweet orange leaves, 118
Cyclic & non-cyclic photophosphorylation in chloroplasts distinguished by use of labeled oxygen, 44
Cytochrome system in *Schizophyllum commune*, 79
Cytochromes, oxidative activity of cytoplasmic particles of apples, 804

—D—

Dalapon in plants, distribution & metabolic fate of, 698
Daucus carota, suspension cultures in synthetic media, 483
DAVIS, R. M. Basis of shoot response to root temperature in tomato, 153
DEGMAN, E. S. See BENSON, N. R., 296
Degradation of *S,S,S*-tributylphosphorotriothioate by cotton leaves, 816
DELWICHE, C. C. Influence of cobalt on nitrogen fixation by Medicago, 73
DNA content of broad bean (*Vicia faba*) internodes in connection with tumor induction by *Agrobacterium tumefaciens*, 216
DNA extractability & its determination in tissues of higher plants, 212
Deoxyribonucleic acid of cotton, 811
DEWEY, L. J. See STEPKA, W., 592
Dianthus barbatus, salt tolerance & protoplasmic salt hardiness of, 478
2,4-Dichlorophenoxyacetic acid effect on utilization of labeled acetate by bean leaf & stem tissues, 192
2,4-Dichlorophenoxyacetic acid effects on soluble nucleotides & nucleic acid of soybean seedlings, 145
2,4-Dichlorophenoxyacetic acid in bean plants, effects of moisture stress on absorption, translocation, & distribution of, 573
2,4-Dichlorophenoxyacetic acid inactivation in bean stem sections, 89
2,4-Dichlorophenoxyacetic acid metabolites from bean stems, 558
Diffusion of tritiated water into roots as influenced by water status of tissue, 331
DILLEY, DAVID R. Assimilation of C^{14}, N^{15} labeled urea by excised apple & peach leaves, 757
Diphosphopyridine nucleotide, reduced, oxidation of by mitochondria from normal & crown-gall tissue cultures of tomato, 347
DPNH oxidase activity, effects of salts on, & structure of sweet potato mitochondria, 445
Distribution of boron in leaves, 420
Dormancy in Betula as a quantitative state, 643
Dormancy in wheat seeds, germination inhibitors related to, 739
DRINKWATER, W. O. See TAYLOR, G. A., 360
DYAR, JAMES J. A relationship between boron & auxin in C^{14} translocation in bean plants, 672
DYBING, C. DEAN. Foliar penetration by chemicals, 169

—E—

EAGLE, H. E. See LAGERWERFF, J. V., 472
Editor's report, 1960, ASPP, 121
Editor's report, 1961, ASPP, 847
Effect of boron on elongation of tomato root tips, 244
Effect of cations on germination & germ tube development of *Puccina coronata* uredospores, 14
Effect of iron chlorosis on protein fractions of corn leaf tissue, 736
Effect of photochemically produced oxidants on growth of Avena coleoptile sections, 326
Effect of root to solution ratio in ion absorption experiments, 62
Effect of sodium bicarbonate on iron absorption by orange seedlings, 52
Effect of soil moisture stress on absorption & translocation of phosphorus applied to leaves of sunflower, 762
Effect of soil oxygen deficit on iron nutrition of orange seedlings, 425
Effect of 2,4-dichlorophenoxyacetic acid on utilization of labeled acetate by bean leaf & stem tissues, 192
Effect of various cations upon absorption of carrier-free cesium, 66
Effects of calcium, potassium, & magnesium on oxalic, malic, & citric acid content of Valencia orange leaf tissue, 99
Effects of ethylene & oxygen on production of a bitter compound by carrot roots, 550
Effects of moisture stress on absorption, translocation, & distribution of 2,4-dichlorophenoxyacetic acid in bean plants, 573
Effects of salts on DPNH oxidase activity & structure of sweet potato mitochondria, 445
Effect of temperature on water transport through plants, 639
Effects of thermoperiodism on tuber formation in *Ipomoea batatas* under controlled conditions, 680
Effects of wax coatings on leaf temperatures & field survival of *Pinus taeda* seedlings, 180
Effects of X irradiation on corn seed, 566
Elaeagnus angustifolia, salt tolerance & protoplasmic salt hardiness of, 478
EMERSON, MERLE T. See ADAMS, DONALD F., 261
Entry of phosphate into yeast cell, 277
Enzymatic activities associated with cell wall preparations from corn coleoptiles, 605
EPSTEIN, EMANUEL. Essential role of calcium in selective cation transport by plant cells, 437
EPSTEIN, SAMUEL. See PARK, RODERIC, 133
ERGLE, DAVID R. Deoxyribonucleic acid of cotton, 811
Essential role of calcium in selective cation transport by plant cells, 437
Estimate of a minimum quantum yield of photosynthesis based on ecologic data, 371
Ethylene effects on production of a bitter compound by carrot roots, 550
Euglena gracilis, reductive pentose phosphate cycle, 409

Euonymus alatus, salt tolerance & protoplasmic salt hardiness of, 478
EVANS, HAROLD J. See LOWE, RICHIE H., 545
EVANS, WM. R. Pyrimidine metabolism in germinating seedlings, 9
EVERSON, E. H. See MIYAMOTO, TAKAO, 739
Executive committee meeting, American Society Plant Physiologists, 1960, 121
Executive committee meeting, American Society Plant Physiologists, 1961, 847
Extractability of DNA & its determination in tissues of higher plants, 212

—F—

FANG, S. C. Effect of 2,4-dichlorophenoxyacetic acid on utilization of labeled acetate by bean leaf & stem tissues, 192
FELLIG, J. See BACH, MICHAEL K., 85, 89
FLESHER, DONNA. See CHERRY, JOE H., 566
Flowering stimulus in Xanthium, persistence & transport of, 656
Fluoride fumigation, variations in starch & total polysaccharide content of *Pinus ponderosa* needles with, 261
Fluoride induced chlorosis, mechanism(s) of, 385
Foliar penetration by chemicals, 169
Formation of auxin from tryptophan through action of polyphenols, 838
Formation of sucrose from malate in germinating castor beans. I. Conversion of malate to phosphoenol-pyruvate, 540
FORREST, H. S. See HATFIELD, D. L., 240
FOY, CHESTER L. Absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity. I. Penetration & translocation of Cl^{36}- and C^{14}-labeled dalapon, 688
FOY, CHESTER L. Absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity. II. Distribution & metabolic fate of dalapon in plants, 698
Fraxinus pennsylvanica, salt tolerance & protoplasmic salt hardiness of, 478
FRIED, MAURICE. Kinetics of rubidium absorption & translocation by barley, 183
Frost hardening, changes in SH content during, 611
Frost resistance, new factor in, 617
Fructose origin in inner space, accumulation & transformation of sugars in stalks of sugar cane, 175
Fruit-induced & apical senescence in *Pisum sativuum*, L., 389
Fruits, developing photosynthesis & respiration in, 69
FULLER, WALLACE H. See SMITH, RICHARD F., 747
Further studies on a particulate enzyme preparation from nodules of soybean plants, 545
Further studies of synergism in slit pea test, 92

—G—

GAIRON, S. See ORDIN, L., 331
GALLY, JOSEPH. See MUIR, ROBT. M., 222
GALSTON, ARTHUR W. See ASOMANING, EDWIN J. A., 453
Gametocide response in cotton, mechanisms & reversal of, 529
GARBER, M. J. See WALLIHAN, E. F., 425
General index, Vol. 36, 851
Geotropic curvature of Avena coleoptiles in presence of *n*-1-naphthylphthalamic acid, 727
Geotropic response mediation by lateral transport of auxin, 364
GERLOFF, GERALD C. See SCHMID, WALTER E., 226
Germ tube development of *Puccinia coronata* uredospores, 14
Germination inhibitors related to dormancy in wheat seeds, 739
Gibberellic acid interaction with allyl trimethylammonium bromide upon growth of Ulothrix, 685
Gibberellic acid, physiological effects of, 829
Gibberellin responses of light-requiring seeds of lettuce & *Lepidium virginicum*, 663
GILLESPIE, BARBARA. Mediation of geotropic response by lateral transport of auxin, 364
GLASZIOU, K. T. Accumulation & transformation of sugars in stalks of sugar cane. Origin of glucose & fructose in the inner space, 175
Gleditsia triacanthos, salt tolerance & protoplasmic salt hardiness of, 478
Glucose origin in inner space, accumulation & transformation of sugars in stalks of sugar cane, 175
Glycine max, carbohydrate translocation in, 820
Glycine max, chlorosis in higher plants as related to organic acid content, 415
Glycine max, mechanism(s) of fluoride induced chlorosis, 385
Glycine max, particulate enzyme preparation from nodules of, 545
Glycine max, selective absorption of iron from iron chelates by, 710
Glycine max, selective translocation of products of photosynthesis in, 581
GOOD, N. E. See KRALL, A. R., 44
GOOD, NORMAN E. Inhibitors of Hill reaction, 788
GORDON, S. A. Formation of auxin from tryptophan through action of polyphenols, 838
GORHAM, PAUL R. See NELSON C. D., 581
Gossypium barbadense, deoxyribonucleic acid of cotton, 811
Gossypium barbadense, mechanisms & reversal of gametocide response in, 529
Gossypium hirsutum, absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity, 688
Gossypium hirsutum, deoxyribonucleic acid of cotton, 811
Gossypium hirsutum, isolation of metabolically-active subcellular particles from etiolated seedling hypocotyls using bovine serum albumin in preparative medium, 302
Gossypium hirsutum, mechanisms & reversal of gametocide response in, 529
GOTTSCHALL, VIRGINIA. See LOCKHART, JAMES A., 389

GRAHAM, HORACE D. Reaction of gibberellic acid & gibberellins with Folin-Wu phosphomolybdic acid reagent & its use for quantitative assay, 405
GRAHAM, JO-RUTH. See MYERS, JACK, 342
GRIGSBY, B. H. See HAMILTON, R. H., 354
Growth & geotropic curvature of Avena coleoptiles in presence of *n*-1-naphthylphthalamic acid, 727
Growth of *Parthenocissus tricuspidata* tissue cultured on media containing aza analogues of indole-3-acetic acid and indole-3-propionic acid, 621
Growth regulators, plant, thiocarbamates as, 222
Growth substances, naturally occurring, 48

— H —

HABER, ALAN H. Pigment formation & photosynthesis in dormant lettuce seeds, 351
HABERMANN, HELEN M. Light dependent oxygen metabolism of chloroplast preparations. III. Photo-oxidation of ascorbic acid, 252
HACKETT, DAVID P. Effects of salts on DPNH oxidase activity & structure of sweet potato mitochondria, 445
HACKETT, DAVID P. See NIEDERPRUEM, DONALD J., 79
HAGEMAN, R. H. See CHERRY, JOE H., 163, 566
HAGEN, CHAS. W., JR. See KLEIN, ATTILA O., 1
HALL, WAYNE C. See KATTERMAN, FRANK R. H., 816
HAMILTON, R. H. Isolation of indole-3-acetic acid from corn kernels & etiolated corn seedlings, 354
HANDLEY, RAYMOND. Effect of various cations upon absorption of carrier-free cesium, 66
HANDLEY, RAYMOND. Uptake of calcium & chlorine in roots of *Zea mays*, 766
HANNAPEL, RAYMOND J. See JACOBSON, LOUIS, 58, 62
HANSCH, CORWIN. See MUIR, ROBT. M., 222
HANSON, JOHN B. See KEY, JOE L., 145
Haplopappus gracilis, suspension cultures in synthetic media, 483
HATFIELD, D. L. Pteridines in blue green algae, 240
Heat injury, translocated, 721
Helianthus annuus, effect of soil moisture stress on absorption & translocation of phosphorus applied to leaves, 762
Helianthus annuus, moisture stress effects on, 573
Helianthus annuus, negative transport & resistance to water flow through plants, 633
HENDERSON, J. H. M. See GRAHAM, HORACE D., 405
HENDRICKS, S. B. See TOOLE, VIVIAN K., 285
Hexitols in coconut milk: Their role in nurture of dividing cells, 492
HIATT, A. J. Preparation & some properties of soluble succinic dehydrogenase from higher plants, 552
HILDEBRANDT, A. C. See TAMAOKI, TAIKI, 347
Hill reaction inhibitors, 788
Hill reaction, stimulation of by carbon dioxide, 202
HOLM, L. G. See TAYLORSON, RAY, 465
HONDA, S. I. Some effects of tonicity on *Lupine succinoxidase*, 105
Hordeum distichon, physiological effects of gibberellic acid, 829
Hordeum vulgare, comparative study of phototropic response & pigment content in coleoptiles, 453
Hordeum vulgare, effect of root to solution ratio in ion absorption experiments, 62
Hordeum vulgare, essential role of calcium in selective cation transport by cells, 437
Hordeum vulgare, influence of calcium on selectivity of ion absorption process, 58
Hordeum vulgare, uptake of calcium by excised roots, 53
HUDSON, ROBT. J. See WIXON, ROBT. L., 598

— I —

Identification & mode of action of component of positively-ionized air causing enhanced growth in plants, 747
Impatiens balsamina, anthocyanin production in detached petals of, 1
Impatiens balsamina, certain properties of hypocotyl reflecting physiological complexity, 650
Indole-3-acetic acid influence on translocation of foliar applied radiocalcium, 360
Indole-3-acetic acid isolation from corn kernels & etiolated corn seedlings, 354
3-Indoleacetic acid metabolism of by pea roots, 783
Indoleacetic acid oxidase inhibitor from pea tips, 752
Influence of calcium on selectivity of ion absorption process, 58
Influence of cobalt on nitrogen fixation by Medicago, 73
Influence of reproductive organs on secretion of sugars in flowers of *Streptosolen jamesonii*, Miers, 265
Influence of 2,3,5-triiodobenzoic acid, indole-3-acetic acid & method of sample collection on translocation of foliar applied radiocalcium, 360
Inhibitor of indoleacetic acid oxidase from pea tips, 752
Inhibitor, invertase, in potato, 313
Inhibitors of Hill reaction, 788
Instructions for contributors to Plant Physiology, 129, 273, 381, 524, 873
Interaction of gibberellic acid & allyl trimethylammonium bromide upon growth of Ulothrix, 685
Invertase & invertase inhibitor in potato, 313
Involvement of adenylate kinase in phosphorylation of ribose-5-phosphate by adenosinediphosphate in mitochondria-free preparations from rough lemon leaves, 368
Ion absorption experiments, effect of root to solution ratio in, 62
Ion absorption process, influence of calcium on selectivity of, 58
Ionized air, component causing enhanced growth in plants, 747

Ipomoea batatas, effects of salts on DPNH oxidase activity & structure of mitochondria, 445
Ipomoea batatas, effects of thermoperiodism on tuber formation under controlled conditions, 680
Ipomeamarone alterations in sweet potato roots infected by *Ceratocystis fimbriata*, 139
Iron absorption by orange seedlings, effect of sodium bicarbonate on, 52
Iron chelate, naturally occurring, in xylem exudate, 226
Iron chlorosis effect on protein fractions of corn leaf tissue, 736
Iron nutrition of orange seedlings, effect of soil oxygen deficit on, 425
Iron, selective absorption of from iron chelates by soybean plants, 710
Isolation & characterization of malformin, 30
Isolation of indole-3-acetic acid from corn kernels & etiolated corn seedlings, 354
Isolation of metabolically-active subcellular particles from etiolated cotton seedling hypocotyls using bovine serum albumin in preparative medium, 302

— JK —

JACKSON, C. F. See WOLTZ, S. S., 197
JACOBSON, LOUIS. Effect of root to solution ratio in ion absorption experiments, 62
JACOBSON, LOUIS. Influence of calcium on selectivity of ion absorption process, 58
JACOBSON, LOUIS. See MOORE, DAVID P., 53, 290
JENSEN, R. DUANE. Effect of temperature on water transport through plants, 639
JENSEN, R. DUANE. Negative transport & resistance to water flow through plants, 633
JOHANSSON, NILS-OLOF. See LEVITT, J., 611
JOHNSON, C. M. See DELWICHE, C. C., 73
Juglans nigra, salt tolerance & protoplasmic salt hardiness of, 478
Juniperus virginiana, salt tolerance & protoplasmic salt hardiness of, 478
KAHN, DAVID. A note on efficiency of photon absorption, 539
KATTERMAN, FRANK R. H. Physiological effects & degradation of *S,S,S*-tributylphosphorotriothioate by cotton leaves as shown by the P^{32} & S^{35} labeled compounds, 816
KATTERMAN, FRANK R. H. See ERGLE, DAVID R., 811
KAWASE, MAKOTO. Dormancy in Betula as a quantitative state, 643
KEFFORD, N. P. Steady state growth of Avena coleoptile sections in high auxin concentrations, 323
KEY, JOE L. Some effects of 2,4-dichlorophenoxyacetic acid on soluble nucleotides & nucleic acid of soybean seedlings, 145
KIM, YONG CHOLL. Effects of thermoperiodism on tuber formation in *Ipomoea batatas* under controlled conditions, 680
Kinetics of rubidium absorption & translocation by barley, 183
Kinetin, rapid bioassay for, using senescing leaf tissue, 219
Kinins, rapid bioassay for, using senescing leaf tissue, 219
KIVILAAN, A. Enzymatic activities associated with cell wall preparations from corn coleoptiles, 605
KLEIN, ATTILA O. Anthocyanin production in detached petals of *Impatiens balsamina* L., 1
KLEIN, WM. H. See PRICE, LEONARD, 733
Kochia scoparia, salt tolerance & protoplasmic salt hardiness of, 478
KOHL, HARRY C. Distribution of boron in leaves, 420
KRALL, A. R. Cyclic & non-cyclic photophosphorylation in chloroplasts distinguished by use of labeled oxygen, 44
Krebs cycle oxidations, oxidative activity of cytoplasmic particles of apples, 804
KUPILA, SIRKKA. DNA content of broad bean (*Vicia faba*) internodes in connection with tumor induction by *Agrobacterium tumefaciens*, 216
KUPILA, SIRKKA. Extractability of DNA & its determination in tissues of higher plants, 212

— L —

Labeled oxygen use, cyclic & non-cyclic photophosphorylation in chloroplasts distinguished by, 44
Lactuca sativa, pigment formation & photosynthesis in dormant seeds, 351
Lactuca scariola, foliar penetration by chemicals, 169
LAGERWERFF, J. V. Osmotic & specific effects of excess salts on beans, 472
Leaf temperatures, effects of wax coatings on, *Pinus taeda* seedlings, 180
LEGGETT, J. E. Entry of phosphate into yeast cell, 277
LEOPOLD, A. C. Auxin-phenol complexes, 589
Lepidium virginicum, responses to gibberellin of light-requiring seeds, 663
LE TOURNEAU, DUANE. Oligosaccharide production from sucrose by *Verticillium albo-atrum*, 102
LEVITT, J. See SCHMUTZ, WALTER, 617
LEVITT, J. Sulfhydryls—A new factor in frost resistance. I. Changes in SH content during frost hardening, 611
LIEBERMAN, MORRIS. Oxidative activity of cytoplasmic particles of apples: Krebs cycle oxidations, oxidative phosphorylation, & cytochromes, 804
Light dependent oxygen metabolism of chloroplast preparations. III. Photooxidation of ascorbic acid, 252
Light responses of seeds of *Pinus virginiana*, 285
Lilium longiflorum, distribution of boron in leaves, 420
LINGLE, J. C. See DAVIS, R. M., 153
LOCKHART, JAMES A. Fruit-induced & apical senescence in *Pisum sativum* L., 389
LOEHWING, WALTER FERDINAND, obituary, 126
Lonicera japonica, salt tolerance & protoplasmic salt hardiness of, 478

LOWE, RICHIE H. Further studies on a particulate enzyme preparation from nodules of soybean plants, 545
Lupinus albus, tonicity effects on, 105
Lycopersicon esculentum, effect of boron on elongation of root tips, 244
Lycopersicon esculentum, basis of shoot response to root temperature in, 153
Lycopersicon esculentum, influence of 2,3,5-triiodobenzoic acid, indole-3-acetic acid & method of sample collection on translocation of foliar applied radiocalcium, 360
Lycopersicon esculentum, metabolic fractionation of C^{13} & C^{12} in plants, 133
Lycopersicon esculentum, negative transport & resistance to water flow through plants, 633

— M —

Magnesium effects on oxalic, malic, & citric acid content of Valencia orange leaf tissue, 99
Magnesium uptake & its interaction with calcium in excised barley roots, 290
MAKOWER, RACHEL U. See SCHWIMMER, SIGMUND, 313
Malate in germinating castor beans, formation of sucrose from, 540
Maleic hydrazide-auxin interactions in water uptake of potato discs, 465
Malic acid content of Valencia orange leaf tissue, effects of calcium, potassium, & magnesium on, 99
Malus sylvestris, oxidative activity of cytoplasmic particles of, 804
MARR, A. G. Rapid cryoscopic technique for measuring osmotic properties of drop size samples, 677
Mass adaptation to selenomethionine in populations of *Chlorella vulgaris*, 502
Mass culture of algae, 342
MAYNE, B. C. See ABELES, FREDRICK B., 202
MAYNE, B. C. See KRALL, A. R., 44
MCCALLA, D. R. See OSBORNE, DAPHNE J., 219
MCKELL, CYRUS M. See WILSON, A. M., 762
MCLEOD, G. C. Action spectra of light-saturated photosynthesis, 114
MCNULTY, I. B. Mechanism(s) of fluoride induced chlorosis, 385
Mechanism of stimulation of respiration by purines: I. Inhibitor studies, 317
Mechanisms & reversal of gametocide response in cotton, 529
Mechanisms by which wind influences transpiration, 112
Mechanism(s) of fluoride induced chlorosis, 385
Mediation of geotropic response by lateral transport of auxin, 364
Medicago sativa, influence of cobalt on nitrogen fixation by, 73
Metabolic fractionation of C^{13} & C^{12} in plants, 133
Metabolites of 2,4-dichlorophenoxyacetic acid from bean stems, 558
MEYER, RAYMOND E. See BASLER, EDDIE, 573
MICHEL, BURLYN E. Further studies of synergism in slit pea test, 92
Microcoleus vaginatus, identification & mode of action of component of positively-ionized air causing enhanced growth in plants, 747
Midwestern section, American Society of Plant Physiologists, 715
MILLER, GENE W. See SU, LUCIA YULIN, 415
Mitochondria from normal & crown-gall tissue cultures of tomato, oxidation of reduced diphosphopyridine nucleotide by, 347
Mitochondria, sweet potato, effects of salts on DPNH oxidase activity & structure of, 445
MIYAMOTO, TAKAO. Germination inhibitors related to dormancy in wheat seeds, 739
Moisture stress effect on absorption & translocation of phosphorus applied to leaves of sunflower, 762
Moisture stress effects on absorption, translocation, & distribution of 2,4-dichlorophenoxyacetic acid in bean plants, 573
MONK, RALPH W. Salt tolerance & protoplasmic salt hardiness of various woody & herbaceous ornamental plants, 478
MOORE, DAVID P. See JACOBSON, LOUIS, 58, 62
MOORE, DAVID P. Uptake of calcium by excised barley roots, 53
MOORE, DAVID P. Uptake of magnesium & its interaction with calcium in excised barley roots, 290
MOORE, J. N. See TAYLOR, G. A., 360
MORTIMER, D. C. See NELSON, C. D., 581
MUELLER, R. T. See WALLACE, A., 118
MUENSTER, ANNE-MARIE. See HONDA, S. I., 105
MUIR, ROBT. M. Thiocarbamates as plant growth regulators, 222
MUMFORD, FRANKLIN E. An inhibitor of indoleacetic acid oxidase from pea tips, 752
Musa acuminata, photosynthesis & transpiration from upper & lower surfaces of intact leaves, 399
Musa acuminata, photosynthesis & transpiration of leaves as affected by severing vascular system, 577
MYERS, JACK. On mass culture of algae. III. Light diffusers: high vs low temperature Chlorellas, 342

— N —

NAKAYAMA, F. S. See BIDDULPH, O., 429
n-1-Naphthylphthalamic acid, growth & geotropic curvature of Avena coleoptiles in presence of, 727
Naturally occurring chelate of iron in xylem exudate, 226
Naturally occurring growth substances. II. An improved straight growth test & its applications, 48
Negative transport & resistance to water flow through plants, 633
NELSON, C. D. Selective translocation of products of photosynthesis in soybean, 581
Neurospora crassa, hexitols in coconut milk, 492
NEVYAS, JOANN. See SHRIFT, A., 502, 509

NEWMAN, D. W. See McNULTY, I. B., 385
NEWS & notes, 121, 359, 520, 715, 847
Nicotiana tabacum, cell wall carbohydrates in tobacco pith parenchyma as affected by boron deficiency & by growth in tissue culture, 336
Nicotiana tabacum, conversion of nicotine to nornicotine in harvested plants: fate of methyl group, 592
Nicotiana tabacum, naturally occurring chelate of iron in xylem exudate, 226
Nicotiana tabacum, preparation & some properties of soluble succinic dehydrogenase from, 552
Nicotine conversion to nornicotine in harvested tobacco, 592
NIEDERPRUEM, DONALD J. Cytochrome system in *Schizophyllum commune*, 79
Nitrogen fixation by Medicago, influence of cobalt on, 73
Nitrogen metabolism changes in sweet potato with black rot, 770
NOGGLE, J C. See FRIED, MAURICE, 183
Nornicotine conversion from nicotine in harvested tobacco, 592
Northeastern section, American Society of Plant Physiologists, 520
NORTON, GRENVILLE. See ROMBERGER, JOHN A., 20
Nostoc muscorum, pteridines in, 240
Note on efficiency of photon absorption, 539
Nucleic acid of soybean seedlings, effects of 2,4-dichlorophenoxyacetic acid on, 145
Nucleotide & ribonucleid acid metabolism of corn seedlings, 163
Nucleotides, soluble, effects of 2,4-dichlorophenoxyacetic acid on soybean seedlings, 145

—O—

Oat, naturally occurring growth substances, 48
OBERLÄNDER, HANS E. See FRIED, MAURICE, 183
OERTLI, J. J. See KOHL, HARRY C., 420
Oligosaccharide production from sucrose by *Verticillium albo-atrum*, 102
On the mass culture of algae. III. Light diffusers: high vs low temperature Chlorellas, 342
ORDIN, L. Diffusion of tritiated water into roots as influenced by water status of tissue, 331
ORDIN, LAWRENCE. Effect of photochemically produced oxidants on growth of Avena coleoptile sections, 326
Organic acid content, chlorosis in higher plants as related to, 415
OSBORNE, DAPHNE J. Rapid bioassay for kinetin & kinins using senescing leaf tissue, 219
OSBORNE, THOS. S. Two improved & inexpensive systems for moisture stabilization in seeds or other tissues, 309
Osmostic & specific effects of excess salts on beans, 472
Osmotic properties of drop size samples, rapid cryoscopic technique for measuring, 677
OVERSTREET, ROY. See HANDLEY, RAYMOND, 66, 766
OVERSTREET, ROY. See MOORE, DAVID P., 53, 290
Oxalic acid content of Valencia orange leaf tissue, effects of calcium, potassium, & magnesium on, 99
Oxidation of reduced diphosphopyridine nucleotide by mitochondria from normal & crown-gall tissue cultures of tomato, 347
Oxidative activity of cytoplasmic particles of apples: Krebs cycle oxidations, oxidative phosphorylation, & cytochromes, 804
Oxidative phosphorylation, oxidative activity of cytoplasmic particles of apples, 804
Oxygen deficit, soil, effect on iron nutrition of orange seedlings, 425
Oxygen effects on production of a bitter compound by carrot roots, 550
Oxygen evolution, time course of during photosynthesis in synchronized cultures of algae, 232
Oxygen metabolism, light dependent, of chloroplast preparations, 252

—PQ—

PALEG, L. G. See GORDON, S. A., 838
PALEG, LESLIE G. Physiological effects of gibberellic acid. III. Observations on its mode of action on barley endosperm, 829
PARK, RODERIC. Metabolic fractionation of C^{13} & C^{12} in plants, 133
Parthenocissus tricuspidata, growth of tissue cultured on media containing aza analogues of indole-3-acetic acid & indole-3-propionic acid, 621
Parthenocissus tricuspidata, ubiquity of stimulation of endogenous respiration by purines & purine analogs, 85
Persea americana, photosynthesis & respiration in developing fruits, 69
Persistence & transport of flowering stimulus in Xanthium, 656
PERUR, N. G. Effect of iron chlorosis on protein fractions of corn leaf tissue, 736
PETERKOFSKY, A. Reductive pentose phosphate cycle. III. Enzyme activities in cell-free extracts of photosynthetic organisms, 409
PETERSON, C. E. See CARLTON, BRUCE, C., 550
PETTIT, ROBT. M. See LEVITT, J., 611
Petunia hybrida, salt tolerance & protoplasmic salt hardiness of, 478
Phaseolus aureus, formation of auxin from tryptophan through action of polyphenols, 838
Phaseolus limensis, critical phosphorus levels for growth, 626
Phaseolus vulgaris, effect of 2,4-dichlorophenoxyacetic acid on utilization of labeled acetate by leaf & stem tissues, 192
Phaseolus vulgaris, foliar penetration by chemicals, 169
Phaseolus vulgaris, mechanism(s) of fluoride induced chlorosis, 385
Phaseolus vulgaris, metabolites of 2,4-dichlorophenoxyacetic acid from stems, 558

Phaseolus vulgaris, osmotic & specific effects of excess salts on, 472
Phaseolus vulgaris, preparation & some properties of soluble succinic dehydrogenase from, 552
Phaseolus vulgaris, red, far-red response in chlorophyll synthesis, 733
Phaseolus vulgaris, relationship between boron & auxin in C^{14} translocation in, 672
Phaseolus vulgaris, response of seedlings to malformin, 37
Phaseolus vulgaris, translocated heat injury, 721
Phaseolus vulgaris, transpiration stream & ascension of calcium, 429
Phenol-auxin complexes, 589
Phormidium persicinum, action spectra of light-saturated photosynthesis, 114
Phosphate entry into yeast cell, 277
P^{32} labeled compounds, physiological effects & degradation of *S,S,S*-tributylphosphorotriothioate as shown by, 816
Phosphorylation, involvement of adenylate kinase in, of ribose-5-phosphate by adenosinediphosphate in mitochondria-free preparations from rough lemon leaves, 368
Photooxidation of ascorbic acid, 252
Photophosphorylation, cyclic & non-cyclic in chloroplasts distinguished by use of labeled oxygen, 44
Photosynthesis & respiration in developing fruits. II. Comparative rates at various stages of development, 69
Photosynthesis & transpiration from upper & lower surfaces of intact banana leaves, 399
Photosynthesis & transpiration of banana leaves as affected by severing the vascular system, 577
Photosynthesis based on ecologic data, estimate of minimum quantum yield of, 371
Photosynthesis in dormant lettuce seeds, 351
Photosynthesis in soybean, selective translocation of products of, 581
Photosynthesis in synchronized cultures of algae, time course of oxygen evolution during, 232
Photosynthesis, light-saturated, action spectra of, 114
Physiological effects & degradation of *S,S,S*-tributylphosphorotriothioate by cotton leaves as shown by the P^{32} & S^{35} labeled compounds, 816
Physiological effects of gibberellic acid. III. Observations on its mode of action on barley endosperm, 829
Physiology Workshop, Southern Forest Tree, 850
Phytolacca americana, light dependent oxygen metabolism of chloroplast preparations, 252
Picea omorika, estimate of minimum quantum yield of photosynthesis based on ecologic data, 371
Picea pungens, salt tolerance & protoplasmic salt hardiness of, 478
Pigment formation & photosynthesis in dormant lettuce seeds, 351
Pinus ponderosa, salt tolerance & protoplasmic salt hardiness of, 478
Pinus ponderosa, variations in starch & total polysaccharide content of with fluoride fumigation, 261
Pinus taeda seedlings, effects of wax coatings on leaf temperatures & field survival of, 180
Pinus virginiana, responses of seeds to light, 285
Pisum sativum, fruit-induced & apical senescence in, 389
Pisum sativum, inhibitor of indoleacetic acid oxidase from tips, 752
Pisum sativum, synergism in, 92
PLUMMER, T. H. See LEOPOLD, A. C., 589
POLLARD, J. K. Hexitols in coconut milk: Their role in nurture of dividing cells, 492
Polysaccharide content of *Pinus ponderosa* needles with fluoride fumigation, 261
Portulaca grandiflora, salt tolerance & protoplasmic salt hardiness of, 478
Potassium effects on oxalic, malic, & citric acid content of Valencia orange leaf tissue, 99
Preparation & some properties of soluble succinic dehydrogenase from higher plants, 552
PRICE, LEONARD. Red, far-red response & chlorophyll synthesis, 733
PRINTY, WILMA L. See WALLIHAN, E. F., 425
Production of yellow strapleaf of chrysanthemum & similar disorders by amino acid treatment, 197
PROPST, BETTY. See ORDIN, LAWRENCE, 326
PROPST, BETTY. See TODD, GLENN W., 69
Protein fractions of corn leaf tissue, iron chlorosis effect on, 736
Prunus armeniaca, foliar penetration by chemicals, 169
Prunus persica, assimilation of C^{14}, N^{15} labeled urea by excised leaves, 757
Prunus persica, translocated heat injury, 721
Pseudotsuga menziesii, salt tolerance & protoplasmic salt hardiness of, 478
Pteridines in blue green algae, 240
Puccinia coronata uredospores, effect of cations on germination & germ tube development of, 14
Purines, stimulation of respiration by, 317
Purines, ubiquity of stimulation of endogenous respiration by, 85
Pyrimidine metabolism in germinating seedlings, 9
Pyrus communis, foliar pentration by chemicals, 169
Pyrus Malus, assimilation of C^{14}, N^{15} labeled urea by excised leaves, 757
Pyrus malus, foliar pentration by chemicals, 169
Quantum yield of photosynthesis based on ecologic data, estimate of minimum, 371

—R—

RACKER, E. See PETERKOFSKY, A., 409
Radiocalcium, foliar applied, influence of 2,3,5-triiodobenzoic acid, indole-3-acetic acid, & method of sample collection on translocation of, 360
Rapid bioassay for kinetin & kinins using senescing leaf tissue, 219
Rapid cryoscopic technique for measuring osmotic properties of drop size samples, 677

RASMUSSEN, G. K. Effects of calicum, potassium, & magnesium on oxalic, malic, & citric acid content of Valencia orange leaf tissue, 99
Reaction of gibberellic acid & gibberellins with Folin-Wu phosphomolybdic acid reagent & its use for quantitative assay, 405
Red, far-red response & chlorophyll synthesis, 733
Reductive pentose phosphate cycle. III. Enzyme activities in cell-free extracts of photosynthetic organisms, 409
REINERT, JAKOB. See TORREY, JOHN G., 483
REISENAUER, H. M. See DELWICHE, C. C., 73
Relationship between boron & auxin in C^{14} translocation in bean plants, 672
Reproductive organs, influence on secretion of sugars in flowers of *Streptosolen, jamesonii*, Miers, 265
Respiration, endogenous, ubiquity of stimulation of by purines and purine analogs, 85
Respiration in developing fruits, 69
Respiration of host tree, western X-little cherry virus effects on, 824
Respiration, stimulation of by purines, 317
Respiratory pathways, changing, in potato tuber slices, 20
Responses of seeds of *Pinus virginiana* to light, 285
Responses to gibberellin of light-requiring seeds of lettuce & *Lepidium virginicum*, 663
Rhus trilobata, salt tolerance & protoplasmic salt hardiness of, 478
Ribonucleic acid metabolism of corn seedlings, 163
RIKER, A. J. See TAMAOKI, TAIKI, 347
ROBISON, MICHAEL M. See ROBSON, HOPE H., 621
Robinia pseudoacacia, salt tolerance & protoplasmic salt hardiness of, 478
ROBINSON, J. R. See ANDREAE, W. A., 783
ROBSON, HOPE H. Growth of *Parthenocissus tricuspidata* tissue cultured on media containing aza analogues of indole-3-acetic acid & indole-3-propionic acid, 621
ROMBERGER, JOHN A. Changing respiratory pathways in potato tuber slices, 20
Root temperature in tomato, basis of shoot response to, 153
Root tips, tomato, boron effect on elongation of, 244
Roots, excised barley, calcium uptake by, 53
ROREM, EDW. S. See SCHWIMMER, SIGMUND, 313
Rosa multiflora, salt tolerance & protoplasmic salt hardiness of, 478
Rubidium absorption kinetics by barley, 183

— S —

Saccharomyces cerevisiae, entry of phosphate into yeast cell, 277
Salix alba, salt tolerance & protoplasmic salt hardiness of, 478
Salix purpurea, salt tolerance & protoplasmic salt hardiness of, 478
Salt tolerance & protoplasmic salt hardiness of various woody & herbaceous ornamental plants, 478
SALTMAN, PAUL. See CONRAD, HERBERT M., 685
SCHAEDLE, MICHAIL. See JACOBSON, LOUIS, 58, 62
Schizophyllum commune, cytochrome system in, 79
SCHMID, WALTER E. Naturally occurring chelate of iron in xylem exudate, 226
SCHMUTZ, WALTER. Sulfhydryls—A new factor in frost resistance. II. Relation between sulfhydryls & relative resistance of 15 wheat varieties, 617
SCHRANK, A. R. Growth & geotropic curvature of Avena coleoptiles in presence of *n*-1-naphthylphthalamic acid, 727
SCHWIMMER, SIGMUND. Invertase & invertase invertase inhibitor in potato, 313
SCOTT, RALPH A., JR. Mechanisms & reversal of gametocide response in cotton, 529
SEARLE, NORMAN E. Persistence & transport of flowering stimulus in Xanthium, 656
Secretary-treasurer's report, 1960, ASPP, 121
Secretary-treasurer's report, 1961, ASPP, 847
Seed responses of *Pinus virginiana* to light, 285
Selective absorption of iron from iron chelates by soybean plants, 710
Selective translocation of products of photosynthesis in soybean, 581
Selenomethionine in *Chlorella vulgaris*, stability & reversibility of adaptation to, 509
Selenomethionine in populations of *Chlorella vulgaris*, mass adaptation to, 502
SEN, S. P. See SEN GUPTA, ARCHANA, 374
SEN GUPTA, ARCHANA. Carbon dioxide fixation by auxin treated tissue, 374
Senescence, apical, in *Pisum sativum* L., 389
SHANTZ, E. M. See POLLARD, J. K., 492
SHARPLESS, R. G. See WALLIHAN, E. F., 425
Shepherdia argentea, salt tolerance & protoplasmic salt hardiness of, 478
SHIVE, JOHN WESLEY, obituary, 125
SHRIFT, A. Mass adaptation to selenomethionine in populations of *Chlorella vulgaris*, 502
SHRIFT, A. Stability & reversibility of adaptation to selenomethionine in *Chlorella vulgaris*, 509
SHUEL, R. W. Influence of reproductive organs on secretion of sugars in flowers of *Streptosolen jamesonii*, Miers, 265
Slit pea test, synergism in, 92
SMITH, DEWEY H. See MUMFORD, FRANKLIN E., 752
SMITH, FREDERICK G. See COUEY, H. MELVIN, 14
SMITH, P. F. See RASMUSSEN, G. K., 99
SMITH, R. S. See PERUR, N. G., 736
SMITH, RICHARD F. Identification & mode of action of component of positively-ionized air causing enhanced growth in plants, 747
SNOW, A. G., JR. See TOOLE, VIVIAN K., 285
Solanum tuberosum, changing respiratory pathways in potato tuber slices, 20
Some effects of tonicity on Lupine succinoxidase, 105
Some effects of 2,4-dichlorophenoxyacetic acid on soluble nucleotides & nucleic acid of soybean seedlings, 145
Sorghum halepense, absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity, 688

Sorghum vulgare, absorption, distribution & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity, 688
SOROKIN, CONSTANTINE. Time course of oxygen evolution during photosynthesis in synchronized cultures of algae, 232
Southern Forest Tree Physiology Workshop, 850
Southern section American Society Plant Physiologists report, 359
Soybean seedlings, 2,4-dichlorophenoxyacetic acid effects on soluble nucleotides & nucleic acid of, 145
SPENCER, ROY, JR. See CROSBY, DONALD G., 48
Spinacia oleracea, stimulation of Hill reaction by carbon dioxide, 202
Spiraea vanhouttei, salt tolerance & protoplasmic salt hardiness of, 478
Stability & reversibility of adaptation to selenomethionine in *Chlorella vulgaris*, 509
STAHMANN, MARK. See URITANI, I., 770
Starch variations & total polysaccharide content of *Pinus ponderosa* needles with fluoride fumigation, 261
Steady state growth of Avena coleoptile sections in high auxin concentrations, 323
STEPKA, W. Conversion of nicotine to nornicotine in harvested tobacco: Fate of methyl group, 592
STERN, HERBERT. See KUPILA, SIRKKA, 212, 216
STEWARD, F. C. See POLLARD, J. K., 492
Stimulation of Hill reaction by carbon dioxide, 202
Streptosolen jamesonii, influence of reproductive organs on secretion of sugars in flowers of, 265
Studies on 3-indoleacetic acid metabolism. VII. Metabolism of radioactive 3-indoleacetic acid by pea roots, 783
Studies on response of bean seedlings & corn roots to malformin, 37
Studies in valine biosynthesis: II. Enzymatic dehydration of α,β-dihydroxyisovaleric acid by plant extracts, 598
SU, LUCIA YULIN. Chlorosis in higher plants as related to organic acid content, 415
Succinic dehydrogenase, soluble, from higher plants, preparation & properties of, 552
Succinoxidase, lupine, effects of tonicity on, 105
Sucrose formation from malate in germinating castor beans, 540
Sucrose, oligosaccharide production from by *Verticillium albo-atrum*, 102
Sugar secretion in flowers of *Streptosolen jamesonii*, Miers, influence of reproductive organs on, 265
Sulfhydryl content changes during frost hardening, 611
Sulfhydryls—A new factor in frost resistance. I. Changes in SH content during frost hardening, 611
Sulfhydryls—A new factor in frost resistance. II. Relation between sylfhydryls & relative resistance of 15 wheat varieties, 617
S^{35} labeled compounds, physiological effects & degradation of *S,S,S*-tributylphosphorotriothioate as shown by, 816
SULLIVAN, CHAS. Y. See LEVITT, J., 611
SULLIVAN, CHAS. Y. See SCHMUTZ, WALTER, 617
Suspension cultures of higher plant cells in synthetic media, 483
Sweet potato roots infected by *Ceratocystis fimbriata*, ipomeamarone & chlorogenic acid alterations in, 139
Symbols & abbreviations, 521, 717, 877
Synchronized cultures of algae, time course of oxygen evolution during photosynthesis in, 232
Systems for moisture stabilization in seeds or other tissues, 309

— T —

TAKAHASHI, NOBUTAKA. Isolation & characterization of malformin, 30
TAMAOKI, TAIKI. Oxidation of reduced diphosphopyridine nucleotide by mitochondria from normal & crown-gall tissue cultures of tomato, 347
Tamarix gallica, salt tolerance & protoplasmic salt hardiness of, 478
TAYLOR, G. A. Influence of 2,3,5-triiodobenzoic acid, indole-3-acetic acid & method of sample collection on translocation of foliar applied radiocalcium, 360
TAYLOR, STERLING A. See JENSEN, R. DUANE, 633, 639
TAYLORSON, RAY. Maleic hydrazide-auxin interractions in water uptake of potato discs, 465
TEENY, FUAD. See FANG, S. C., 192
THAMES, JOHN L. Effects of wax coatings on leaf temperatures & field survival of *Pinus taeda* seedlings, 180
Thermoperiodism effects on tuber formation in *Ipomoea batatas* under controlled conditions, 680
Thiocarbamates as plant growth regulators, 222
THOMPSON, JOHN F. See BAKER, JAMES E., 208
THRONEBERRY, GLYN O. Isolation of metabolically-active subcellular particles from etiolated cotton seedling hypocotyls using bovine serum albumin in preparative medium, 302
TIFFIN, LEE O. Selective absorption of iron from iron chelates by soybean plants, 710
Tilia cordata, salt tolerance & protoplasmic salt hardiness of, 478
Time course of oxygen evolution during photosynthesis in synchronized cultures of algae, 232
Tissue culture, cell wall carbohydrates in tobacco pith parenchyma as affected by boron deficiency & by growth in, 336
Tissue cultures, crown-gall, of tomato, oxidation of reduced diphosphopyridine nucleotide by mitochondria from, 347
TODD, GLENN W. Photosynthesis & respiration in developing fruits. II. Comparative rates at various stages of development, 69
TODD, GLENN W. See BASLER, EDDIE, 573
TOLBERT, N. E. See CARLTON, BRUCE C., 550
TOLBERT, N. E. See MIYAMOTO, TAKAO, 739
Tonicity effects on lupine succinoxidase, 105

TOOLE, E. H. See TOOLE, VIVIAN K., 285
TOOLE, VIVIAN K. Responses of seeds of *Pinus virginiana* to light, 285
TOOLE, VIVIAN K. Responses to gibberellin of light-requiring seeds of lettuce & *Lepidium virginicum*, 663
TORREY, JOHN G. Suspension cultures of higher plant cells in synthetic media, 483
Translocated heat injury, 721
Translocation & re-use of boron in broccoli, 296
Translocation by barley, kinetics of, 183
Translocation of C^{14} in bean plants, relationship between boron & auxin in, 672
Translocation of carbohydrate in raspberry & soybean, 820
Translocation of foliar applied radiocalcium, influence of 2,3,5-triiodobenzoic acid, indole-3-acetic acid, & method of sample collection on, 360
Translocation of phosphorus applied to leaves of sunflower, effect of soil moisture stress on, 762
Translocation, selective, of products of photosynthesis in soybean, 581
Transpiration from upper & lower surfaces of intact banana leaves, 399
Transpiration, mechanisms by which wind influences, 112
Transpiration of banana leaves as affected by severing the vascular system, 577
Transpiration stream & ascension of calcium, 429
Tree Physiology Workshop, Southern Forest, 850
S,S,S-tributylphosphorotriothioate degradation by cotton leaves, 816
2,3,5-Triiodobenzoic acid influence on translocation of foliar applied radiocalcium, 360
Tritiated water diffusion into roots as influenced by water status of tissue, 331
Triticum vulgare, germination inhibitors related to dormancy in seeds, 739
Tryptophan, formation of auxin from, through action of polyphenols, 838
Tuber formation in *Ipomoea batatas* under controlled conditions, effects of thermoperiodism, 680
Tumor induction by *Agrobacterium tumefaciens*, DNA content of broad bean internodes in connection with, 216
TURNDORF, SIETSKE. See SHRIFT, A., 502, 509
Two improved & inexpensive systems for moisture stabilization in seeds or other tissues, 309

— U V —

Ubiquity of stimulation of endogenous respiration by purines & purine analogs, 85
Ulothrix substillisima, interaction of gibberellic acid & allyl trimethylammonium bromide upon growth of, 685
ULRICH, ALBERT. Critical phosphorus levels for lima bean growth, 626
Uptake of calcium & chlorine in roots of *Zea mays*, 766
Uptake of calcium by excised barley roots, 53
Uptake of magnesium & its interaction with calcium in excised barley roots, 290
URITANI, I. Changes in nitrogen metabolism in sweet potato with black rot, 770
Uromyces phaseoli, translocated heat injury, 721

— V —

VAADIA, YOASH, See MARR, A. G., 677
Valine biosynthesis, 598
VAN BAALEN, C. See HATFIELD, D. L., 240
VAN NOORT, D. Involvement of adenylate kinase in phosphorylation of ribose-5-phosphate by adenosinediphosphate in mitochondria-free preparations from rough lemon leaves, 368
VAN YSSELSTEIN, M. W. H. See ANDREAE, W. A., 783
Variations in starch & total polysaccharide content of Pinus ponderosa needles with fluoride fumigation, 261
Verticillium albo-atrum, oligosaccharide production from sucrose by, 102
Vicia faba, diffusion of tritiated water into roots as influenced by water status of tissue, 331
Vicia faba, DNA content of internodes in connection with tumor induction by *Agrobacterium tumefaciens*, 216

— W —

WADA, K. See AKAZAWA, T., 139
WALKER, DAVID R. See DILLEY, DAVID R., 757
WALLACE, A. Cyanide & chelating agent effects on in vitro CO_2 fixation in sweet orange leaves, 118
WALLACE, A. See VAN NOORT, D., 368
WALLIHAN, E. F. Effect of soil oxygen deficit on iron nutrition of orange seedlings, 425
WALLIHAN, ELLIS F. Effect of sodium bicarbonate on iron absorption by orange seedlings, 52
Water transport through plants, effect of temperature on, 639
Water uptake of potato discs, maleic hydrazide-auxin interactions in, 465
Wax coating effects on leaf temperatures & field survival of *Pinus taeda* seedlings, 180
WEBB, KENNETH L. See DYAR, JAMES J., 672
Western X-little cherry virus effects on respiration of host tree, 824
WIEBE, H. H. See JENSEN, R. DUANE, 633
WIEBE, HERMAN H. See MONK, RALPH W., 478
WIEBE, HERMAN H. See PERUR, N. G., 736
WILSON, A. M. Effect of soil moisture stress on absorption & translocation of phosphorus applied to leaves of sunflower, 762
WILSON, CURTIS M. Cell wall carbohydrates in tobacco pith parenchyma as affected by boron deficiency & by growth in tissue culture, 336
WILSON, CURTIS M. See ALBERT, LUKE S., 244
Wind, mechanisms of influence on transpiration, 112
WIXOM, ROBT. L. Studies in valine biosynthesis: II. Enzymatic dehydration of α,β-dihydroxisovaleric acid by plant extracts, 598

WOLTZ, S. S. Production of yellow strapleaf of chrysanthemum & similar disorders by amino acid treatment, 197
WOOLLEY, Jos. T. Mechanisms by which wind influences transpiration, 112

—XYZ—

Xanthium pennsylvanicum, rapid bioassay for kinetin & kinins using senescing leaf tissue, 219
Xanthium pennsylvanicum, persistence & transport of flowering stimulus in, 656
X-irradiation effects on corn seed, 566
Xylem exudate, naturally occurring iron chelate in, 226
YARWOOD, C. E. Translocated heat injury, 721
Yeast cell, entry of phosphate into, 277
YOST, HENRY T., JR. See ROBSON, HOPE H., 621
Zea mays, diffusion of tritiated water into roots as influenced by water status of tissue, 331
Zea mays, effect of iron chlorosis on protein fractions of leaf tissue, 736
Zea mays, effects of X irradiation on seed, 566
Zea mays, isolation of indole-3-acetic acid from kernels & etiolated seedlings, 354
Zea mays, mediation of geotropic response by lateral transport of auxin, 364
Zea mays, nucleotide & ribonucleic acid metabolism of seedlings, 163
Zea mays, response of roots to malformin, 37
Zea mays, uptake of calcium & chlorine in roots of, 766
Zebrina pendula, foliar penetration by chemicals, 169
Zinnia elegans, salt tolerance & protoplasmic salt hardiness of, 478

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TABLE OF CONTENTS

NUMBER 1. JANUARY

Anthocyanin production in detached petals of *Impatiens balsimina* L.
ATTILA O. KLEIN & CHARLES W. HAGEN, JR. 1– 9

Pyrimidine metabolism in germinating seedlings.
WILLIAM R. EVANS & BERNARD AXELROD 9– 13

Effect of cations on germination & germ tube development of *Puccinia coronata* uredospores. ..... H. MELVIN COUEY & FREDERICK G. SMITH 14– 19

Changing respiratory pathways in potato tuber slices.
JOHN A. ROMBERGER & GRENVILLE NORTON 20– 29

Isolation & characterization of malformin.
NOBUTAKA TAKAHASHI & ROY W. CURTIS 30– 36

Studies on response of bean seedlings & corn roots to malformin.
ROY W. CURTIS 37– 43

Cyclic and non-cyclic photophosphorylation in chloroplasts distinguished by use of labeled oxygen. ..... A. R. KRALL, N. E. GOOD, & B. C. MAYNE 44– 47

Naturally occurring growth substances. II. An improved straight growth test & its applications.
DONALD G. CROSBY, ROBERT V. BERTHOLD, & ROY SPENCER, JR. 48– 51

Effect of sodium bicarbonate on iron absorption by orange seedlings.
ELLIS F. WALLIHAN 52– 53

Uptake of calcium by excised barley roots.
DAVID P. MOORE, LOUIS JACOBSON, & ROY OVERSTREET 53– 57

Influence of calcium on selectivity of ion absorption process.
LOUIS JACOBSON, RAYMOND J. HANNAPEL, DAVID P. MOORE, & MICHAIL SCHAEDLE 58– 61

Effect of root to solution ratio in ion absorption experiments.
LOUIS JACOBSON, RAYMOND J. HANNAPEL, MICHAIL SCHAEDLE, & DAVID P. MOORE 62– 65

Effect of various cations upon absorption of carrier-free cesium.
RAYMOND HANDLEY & ROY OVERSTREET 66– 69

Photosynthesis & respiration in developing fruits. II. Comparative rates at various stages of development.
GLENN W. TODD, ROSS C. BEAN, & BETTY PROPST 69– 73

Influence of cobalt on nitrogen fixation by Medicago.
C. C. DELWICHE, C. M. JOHNSON, & H. M. REISENAUER 73– 78

Cytochrome system in *Schizophyllum commune*.
DONALD J. NIEDERPRUEM & DAVID P. HACKETT 79– 84

Ubiquity of stimulation of endogenous respiration by purines & purine analogs.
MICHAEL K. BACH & J. FELLIG 85– 88

Correlation between inactivation of 2,4-dichlorophenoxyacetic acid & cessation of callus growth in bean stem sections. .. MICHAEL K. BACH & J. FELLIG 89– 91

Further studies of synergism in slit pea test. ..... BURLYN E. MICHEL 92– 98

Effects of calcium, potassium, & magnesium on oxalic, malic, & citric acid content of Valencia orange leaf tissue. .. G. K. RASMUSSEN & P. F. SMITH 99–101

Oligosaccharide production from sucrose by *Verticillium albo-atrum*.
DUANE LE TOURNEAU 102–104

Some effects of tonicity on lupine succinoxidase.
S. I. HONDA & ANNE-MARIE MUENSTER 105–112

Mechanisms by which wind influences transpiration. JOSEPH T. WOOLLEY 112–114

Action spectra of light-saturated photosynthesis. ..... G. C. MCLEOD 114–117

Cyanide & chelating agent effects on in vitro CO_2 fixation in sweet orange leaves. ..... A. WALLACE & R. T. MUELLER 118–120

News & notes 121–127

Instructions for contributors to Plant Physiology 129–132

Number 2. March

Metabolic fractionation of C^{13} & C^{12} in plants.
Roderic Park & Samuel Epstein 133–138
Analytical study of ipomeamarone & chlorogenic acid alterations in sweet potato roots infected by *Ceratocystis fimbriata*. T. Akazawa & K. Wada 139–144
Some effects of 2,4-dichlorophenoxyacetic acid on soluble nucleotides & nucleic acid of soybean seedlings. Joe L. Key & John B. Hanson 145–152
Basis of shoot response to root temperature in tomato.
R. M. Davis & J. C. Lingle 153–162
Nucleotide & ribonucleic acid metabolism of corn seedlings.
Joe H. Cherry & R. H. Hageman 163–168
Foliar penetration by chemicals. C. Dean Dybing & H. B. Currier 169–174
Accumulation & transformation of sugars in stalks of sugar cane. Origin of glucose & fructose in the inner space. K. T. Glaziou 175–179
Effects of wax coatings on leaf temperatures & field survival of *Pinus taeda* seedlings. John L. Thames 180–182
Kinetics of rubidium absorption & translocation by barley.
Maurice Fried, Hans E. Oberländer, & J C Noggle 183–191
Effect of 2,4-dichlorophenoxyacetic acid on utilization of labeled acetate by bean leaf & stem tissues. S. C. Fang, Fuad Teeny, & Joseph S. Butts 192–196
Production of yellow strapleaf of chrysanthemum & similar disorders by amino acid treatment. S. S. Woltz & C. R. Jackson 197–201
Stimulation of the Hill reaction by carbon dioxide.
Fredrick B. Abeles, A. H. Brown, & B. C. Mayne 202–207
Assimilation of ammonia by nitrogen-starved cells of *Chlorella vulgaris*.
James E. Baker & John F. Thompson 208–212
Extractability of DNA & its determination in tissues of higher plants.
Sirkka Kupila, A. M. Bryan, & Herbert Stern 212–215
DNA content of broad bean (*Vicia faba*) internodes in connection with tumor induction by *Agrobacterium tumefaciens*.
Sirkka Kupila & Herbert Stern 216–219
Rapid bioassay for kinetin & kinins using senescing leaf tissue.
Daphne J. Osborne & D. R. McCalla 219–221
Thiocarbamates as plant growth regulators.
Robert M. Muir, Corwin Hansch, & Joseph Gally 222–225
A naturally occurring chelate of iron in xylem exudate.
Walter E. Schmid & Gerald C. Gerloff 226–231
Time course of oxygen evolution during photosynthesis in synchronized cultures of algae. Constantine Sorokin 232–239
Pteridines in blue green algae.
D. L. Hatfield, C. van Baalen, & H. S. Forrest 240–243
Effect of boron on elongation of tomato root tips.
Luke S. Albert & Curtis M. Wilson 244–251
Light dependent oxygen metabolism of chloroplast preparations. III. Photo-oxidation of ascorbic acid. Helen M. Habermann 252–261
Variations in starch & total polysaccharide content of *Pinus ponderosa* needles with fluoride fumigation. Donald F. Adams & Merle T. Emerson 261–265
Influence of reproductive organs on secretion of sugars in flowers of *Streptosolen jamesonii*, Miers. R. W. Shuel 265–271
Instructions for contributors to Plant Physiology 273–276

Number 3. May

Entry of phosphate into yeast cell. J. E. Leggett 277–284
Responses of seeds of *Pinus virginiana* to light.
Vivian K. Toole, E. H. Toole, S. B. Hendricks, H. A. Borthwick & A. G. Snow, Jr. 285–290

Uptake of magnesium & its interaction with calcium in excised barley roots. DAVID P. MOORE, ROY OVERSTREET, & LOUIS JACOBSON 290–295
Translocation & re-use of boron in broccoli. N. R. BENSON, E. S. DEGMAN, & I. C. CHMELIR 296–301
Isolation of metabolically-active subcellular particles from etiolated cotton seedling hypocotyls using bovine serum albumin in preparative medium. GLYN O. THRONEBERRY 302–309
Two improved & inexpensive systems for moisture stabilization in seeds or other tissues. ..... THOMAS S. OSBORNE & JAMES A. BACON 309–312
Invertase & invertase inhibitor in potato. SIGMUND SCHWIMMER, RACHEL U. MAKOWER, & EDWARD S. ROREM 313–316
Mechanism of stimulation of respiration by purines: I. Inhibitor studies. MICHAEL K. BACH 317–322
Steady state growth of Avena coleoptile sections in high auxin concentrations. N. P. KEFFORD & JAMES BONNER 323–325
Effect of photochemically produced oxidants on growth of Avena coleoptile sections. ..... LAWRENCE ORDIN & BETTY PROPST 326–330
Diffusion of tritiated water into roots as influenced by water status of tissue. L. ORDIN & S. GAIRON 331–335
Cell wall carbohydrates in tobacco pith parenchyma as affected by boron deficiency & by growth in tissue culture. ..... CURTIS M. WILSON 336–341
On the mass culture of algae. III. Light diffusers: high vs. low temperature Chlorellas. ..... JACK MYERS & JO-RUTH GRAHAM 342–346
Oxidation of reduced diphosphopyridine nucleotide by mitochondria from normal & crown-gall tissue cultures of tomato. TAIKI TAMAOKI, A. C. HILDEBRANDT, R. H. BURRIS, & A. J. RIKER 347–351
Pigment formation & photosynthesis in dormant lettuce seeds. ALAN H. HABER & WILLIAM L. CARRIER 351–353
Isolation of indole-3-acetic acid from corn kernels & etiolated corn seedlings. R. H. HAMILTON, R. S. BANDURSKI, & B. H. GRIGSBY 354–359
Influence of 2,3,5-triiodobenzoic acid, indole-3-acetic acid & method of sample collection on translocation of foliar applied radiocalcium. G. A. TAYLOR, J. N. MOORE, & W. O. DRINKWATER 360–363
Mediation of geotropic response by lateral transport of auxin. BARBARA GILLESPIE & WINSLOW R. BRIGGS 364–368
Involvement of adenylate kinase in phosphorylation of ribose-5-phosphate by adenosinediphosphate in mitochondria-free preparations from rough lemon leaves. ..... D. VAN NOORT & A. WALLACE 368–370
An estimate of a minimum quantum yield of photosynthesis based on ecologic data. ..... J. R. BRAY 371–373
Carbon dioxide fixation by auxin treated tissues. ARCHANA SEN GUPTA & S. P. SEN 374–380
News & notes 359
Instructions for contributors to Plant Physiology 381–384

NUMBER 4. JULY

Mechanism(s) of fluoride induced chlorosis. I. B. MCNULTY & D. W. NEWMAN 385–388
Fruit-induced & apical senescence in *Pisum sativum* L. JAMES A. LOCKHART & VIRGINIA GOTTSCHALL 389–398
Photosynthesis & transpiration from upper & lower surfaces of intact banana leaves. ..... WILLIAM A. BRUN 399–405
Reaction of gibberellic acid & gibberellins with Folin-Wu phosphomolybdic acid reagent & its use for quantitative assay. HORACE D. GRAHAM & J. H. M. HENDERSON 405–408
The reductive pentose phosphate cycle. III. Enzyme activities in cell-free extracts of photosynthetic organisms. .. A. PETERKOFSKY & E. RACKER 409–414

Chlorosis in higher plants as related to organic acid content.
LUCIA YULIN SU & GENE W. MILLER 415–420
Distribution of boron in leaves ..... HARRY C. KOHL, JR. & J. J. OERTLI 420–424
Effect of soil oxygen deficit on iron nutrition of orange seedlings.
E. F. WALLIHAN, M. J. GARBER, R. G. SHARPLESS, & WILMA L. PRINTY 425–428
Transpiration stream & ascension of calcium.
O. BIDDULPH, F. S. NAKAYAMA, & R. CORY 429–436
The essential role of calcium in selective cation transport by plant cells.
EMANUEL EPSTEIN 437–444
Effects of salts on DPNH oxidase activity & structure of sweet potato mitochondria. ..... DAVID P. HACKETT 445–452
Comparative study of phototropic response & pigment content in oat & barley coleoptiles. ..... EDWIN J. A. ASOMANING & ARTHUR W. GALSTON 453–464
Maleic hydrazide-auxin interractions in water uptake of potato discs.
RAY TAYLORSON & L. G. HOLM 465–471
Osmotic & specific effects of excess salts on beans.
J. V. LAGERWERFF & H. E. EAGLE 472–477
Salt tolerance & protoplasmic salt hardiness of various woody & herbaceous ornamental plants. ..... RALPH W. MONK & HERMAN H. WIEBE 478–482
Suspension cultures of higher plant cells in synthetic media.
JOHN G. TORREY & JAKOB REINERT 483–491
Hexitols in coconut milk: Their role in nurture of dividing cells.
J. K. POLLARD, E. M. SHANTZ, & F. C. STEWARD 492–501
Mass adaptation to selenomethionine in populations of *Chlorella vulgaris*.
A. SHRIFT, JOANN NEVYAS, & SIETSKE TURNDORF 502–509
Stability & reversibility of adaptation to selenomethionine in *Chlorella vulgaris*.
A. SHRIFT, JOANN NEVYAS, & SIETSKE TURNDORF 509–519
News & notes 520
Symbols & abbreviations commonly used in Plant Physiology 521–523
Instructions for contributors to Plant Physiology 524–527

NUMBER 5. SEPTEMBER

Mechanisms & reversal of gametocide response in cotton.
RALPH A. SCOTT, JR. 529–538
A note on efficiency of photon absorption. ..... DAVID KAHN 539–540
Formation of sucrose from malate in germinating castor beans. I. Conversion of malate to phosphoenol-pyruvate.
C. R. BENEDICT & HARRY BEEVERS 540–544
Further studies on a particulate enzyme preparation from nodules of soybean plants. ..... RICHIE H. LOWE & HAROLD J. EVANS 545–549
Effects of ethylene & oxygen on production of a bitter compound by carrot roots.
BRUCE C. CARLTON, C. E. PETERSON, & N. E. TOLBERT 550–552
Preparation & some properties of soluble succinic dehydrogenase from higher plants. ..... A. J. HIATT 552–557
Metabolites of 2,4-dichlorophenoxyacetic acid from bean stems.
MICHAEL K. BACH 558–565
Effects of X irradiation on corn seed.
JOE H. CHERRY, R. H. HAGEMAN, FLOYD I. COLLINS, & DONNA FLESHER 566–572
Effects of moisture stress on absorption, translocation, & distribution of 2,4-dichlorophenoxyacetic acid in bean plants.
EDDIE BASLER, GLENN W. TODD, & RAYMOND E. MEYER 573–576
Photosynthesis & transpiration of banana leaves as affected by severing the vascular system. ..... WILLIAM A. BRUN 577–580
Selective translocation of products of photosynthesis in soybean.
C. D. NELSON, HEINZ CLAUSS, D. C. MORTIMER, & PAUL R. GORHAM 581–588
Auxin-phenol complexes. ..... A. C. LEOPOLD & T. H. PLUMMER 589–592
Conversion of nicotine to nornicotine in harvested tobacco: Fate of the methyl group. ..... W. STEPKA & L. J. DEWEY 592–597

Studies in valine biosynthesis: II. Enzymatic dehydration of α,β-dihydroxy-isovaleric acid by plant extracts. Robert L. Wixom & Robert J. Hudson 598–604
Enzymatic activities associated with cell wall preparations from corn coleoptiles. A. Kivilaan, T. Cabrera Beaman, & R. S. Bandurski 605–610
Sulfhydryls—a new factor in frost resistance. I. Changes in SH content during frost hardening. J. Levitt, Charles Y. Sullivan, Nils-Olof Johansson, & Robert M. Pettit 611–616
Sulfhydryls—a new factor in frost resistance. II. Relation between sulfhydryls & relative resistance of 15 wheat varieties. Walter Schmutz, Charles Y. Sullivan, & J. Levitt 617–620
Growth of *Parthenocissus tricuspidata* tissue cultured on media containing aza analogues of indole-3-acetic acid & indole-3-propionic acid. Hope H. Robson, Henry T. Yost, Jr., & Michael M. Robison 621–625
Critical phosphorus levels for lima bean growth. Albert Ulrich & Wade L. Berry 626–632
Negative transport & resistance to water flow through plants. R. Duane Jensen, Sterling A. Taylor, & H. H. Wiebe 633–638
Effect of temperature on water transport through plants. R. Duane Jensen & Sterling A. Taylor 639–642
Dormancy in Betula as a quantitative state. Makoto Kawase 643–649
Certain properties of hypocotyl of *Impatiens balsamina* reflecting physiological complexity. Allan Arnold & Ralph E. Alston 650–656
Persistence & transport of flowering stimulus in Xanthium. Norman E. Searle 656–662
Responses to gibberellin of light-requiring seeds of lettuce & *Lepidium virginicum*. Vivian K. Toole & H. M. Cathey 663–671
A relationship between boron & auxin in C^{14} translocation in bean plants. James J. Dyar & Kenneth L. Webb 672–676
Rapid cryoscopic technique for measuring osmotic properties of drop size samples. A. G. Marr & Yoash Vaadia 677–680
Effects of thermoperiodism on tuber formation in *Ipomoea batatas* under controlled conditions. Yong Choll Kim 680–684
Interaction of gibberellic acid & allyl trimethylammonium bromide upon growth of Ulothrix. Herbert M. Conrad & Paul Saltman 685–687
Absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity. I. Penetration & translocation of Cl^{36}- and C^{14}-labeled dalapon. Chester L. Foy 688–697
Absorption, distribution, & metabolism of 2,2-dichloropropionic acid in relation to phytotoxicity. II. Distribution & metabolic fate of dalapon in plants. Chester L. Foy 698–709
Selective absorption of iron from iron chelates by soybean plants. Lee O. Tiffin & J. C. Brown 710–714
News & notes 715
Symbols & abbreviations 717–719

Number 6. November

Translocated heat injury C. E. Yarwood 721–726
Growth & geotropic curvature of Avena coleoptiles in presence of *n*-1-naphthylphthalamic acid. A. R. Shrank 727–732
Red, far-red response & chlorophyll synthesis. Leonard Price & William H. Klein 733–735
Effect of iron chlorosis on protein fractions of corn leaf tissue. N. G. Perur, R. L. Smith, & Herman H. Wiebe 736–739
Germination inhibitors related to dormancy in wheat seeds. Takao Miyamoto, N. E. Tolbert, & E. H. Everson 739–746
Identification & mode of action of a component of positively-ionized air causing enhanced growth in plants. Richard F. Smith & Wallace H. Fuller 747–751

An inhibitor of indoleacetic acid oxidase from pea tips.
FRANKLIN E. MUMFORD, DEWEY H. SMITH, & JOHN E. CASTLE 752–756
Assimilation of C^{14}, N^{15} labeled urea by excised apple & peach leaves.
DAVID R. DILLEY & DAVID R. WALKER 757–761
Effect of soil moisture stress on absorption & translocation of phosphorus applied to leaves of sunflower. ..... A. M. WILSON & CYRUS M. MCKELL 762–765
Uptake of calcium & chlorine in roots of *Zea mays*.
RAYMOND HANDLEY & ROY OVERSTREET 766–769
Changes in nitrogen metabolism in sweet potato with black rot.
I. URITANI & MARK STAHMANN 770–782
Studies on 3-indoleacetic acid metabolism. VII. Metabolism of radioactive 3-indoleacetic acid by pea roots.
W. A. ANDREAE, J. R. ROBINSON, & M. W. H. VAN YSSELSTEIN 783–787
Inhibitors of the Hill reaction ..... NORMAN E. GOOD 788–803
Oxidative activity of cytoplasmic particles of apples: Krebs cycle oxidations, oxidative phosphorylation, & cytochromes. ..... MORRIS LIEBERMANN 804–810
Deoxyribonucleic acid of cotton.
DAVID R. ERGLE & FRANK R. H. KATTERMAN 811–815
Physiological effects & degradation of *S,S,S*-tributylphosphorotriothioate by cotton leaves as shown by the P^{32} & S^{35} labeled compounds
FRANK R. H. KATTERMAN & WAYNE C. HALL 816–819
Carbohydrate translocation in raspberry & soybean.
J. WILLIAM A. BURLEY 820–824
Western X-little cherry virus effects on respiration of host tree.
R. O. BELKENGREN 824–828
Physiological effects of gibberellic acid. III. Observations on its mode of action on barley endosperm ..... LESLIE G. PALEG 829–837
Formation of auxin from tryptophan through action of polyphenols.
S. A. GORDON & L. G. PALEG 838–845
News & Notes 847–850
General Index 851–864
Table of Contents, Annual 867–872
Instructions for contributors to Plant Physiology 873–876
Symbols & Abbreviations 877–879

SUPPLEMENT. i-liv

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6. Mounting. The placement of figures is the author's responsibility; the engraver does not alter the arrangement. Mount all illustrations with rubber cement on white cardboard or stiff white paper. Total size should not exceed 9 x 11 inches. Anything larger than an ordinary file folder is easily mutilated; it also requires special packaging and handling.

7. Identification. Place the author's name on the back of each figure and legend. Place the figure number on the back of each figure. Identification insures against misplacement.

V. SUBMITTING MANUSCRIPTS.

Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown; Department of Botany; University of Minnesota; Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

PLANT PHYSIOLOGY does not accept a manuscript which has not been submitted directly by an author. If your manuscript is submitted by someone other than one of the authors, send a letter to this office stating that you wish to publish that particular manuscript in PLANT PHYSIOLOGY. Your manuscript cannot be processed until this letter is received. This precaution protects you as well as the Journal.

VI. PROCESSING MANUSCRIPT.

A. REVIEW. The manuscript usually will be sent to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

B. REVISION. Manuscripts returned to the author for revision should be sent back to the Editor within two months; otherwise the paper will be published under the date of receipt of the revised manuscript and not the original date.

Retype each page on which there are extensive changes.

C. GALLEY PROOF. This will be sent to the person designated on page 1 above the title. (See II D 1).

1. Returning Proofs. Return only one set of galley proofs to Editor. Keep any other set for reference. Be certain to notify Editor promptly of change of address, so galleys can be sent to the correct address.

2. Charges for Errors. You will be charged at cost for all changes or corrections in galley and page proof which are your fault. You will not be billed for errors which are not your fault. Here are two ways to minimize charges:

a. Proofread Manuscript. Check ALL references carefully BEFORE submitting manuscript to Editor. Corrections in typing are easy to make, whereas corrections in galley or page proof are expensive and sometimes difficult to make.

b. Corrections. PLANT PHYSIOLOGY charges 45c for correcting a straight line of copy; 90c for a line of literature citation or table, $1.80 for a line of formula. Where a correction is optional remember that a change made near the beginning of a paragraph can necessitate resetting every subsequent line to the end of the paragraph. You will be billed for each line the printer must reset to correct an error (this may be a dozen lines), so weigh your cost against the importance of each change you propose.

D. DELAYS. In general, a manuscript should be reviewed and returned to you with the Editor's decision within two months. After you send a revised manuscript to the Editor allow 2½ months for galleys to be sent you for correcting. Should you find that processing your manuscript has taken an unusually long time at either stage, it would be well to inquire of the Editor as to the cause of the delay. (This is a precaution against unrecognized loss of manuscripts in the mail—a rare event, but it happens.)

E. ERRATA. If necessary, an errata will be published for each volume. Authors therefore are requested to call the attention of the Editor to any significant errors in their published manuscripts.

SYMBOLS AND ABBREVIATIONS

COMMONLY USED IN PLANT PHYSIOLOGY

General Rules for Abbreviations

Spell Out

- Words in title & summary (except common chemical symbols & exceptions noted below)
- Words & numerals which begin a sentence
- Units of time (except in tables & parentheses): 30 mg, *but* 50 seconds

Abbreviate

- In parentheses & tables (where possible): (fig 4), (dry wt)
- Units of measurement in text (only when preceded by numeral)

Symbols & Abbreviations*

A-B .....about (*circa*)	ca.
absolute	abs
absorbancy	*A*
adenosine diphosphate	ADP
adenosine triphosphate	ATP
and	& (in parentheses, tables, titles)
and others (*et alii*)	et al.
Ångstrom units(s) (10^{-8} cm)	A (*not* Å)
approximate	approx (in tables)
aqueous	aq
atmosphere(s)	atm
average	avg (in tables)

(*Continued on next page*)

* Abbreviations (with a few exceptions) follow the "Style Manual for Biological Journals," 1960, American Institute of Biological Sciences (Library of Congress Catalog No. 60-15133).

SYMBOLS & ABBREVIATIONS . . . . . .

Continued from page 877

C	centimeter(s)	cm
	coenzyme A (& its acyl derivatives)	CoA
	concentration	conc (in tables)
	counts per minute	cpm
	cubic centimeter(s)	cc (or cm^3)
	curie(s)	c
D-E	degree(s), centigrade	C (omit degree symbol)
	deoxyribonucleic acid	DNA
	diameter	diam (in tables)
	diphosphopyridine nucleotide	DPN
	disintegrations per second	dps
	dry weight	dry wt (in tables)
F	figure	fig (in parentheses & tables)
	-fold	threefold, manifold, *but* 12-fold
	foot candle(s)	ft-c
	fresh weight	fr wt (in tables)
G	gram(s)	g
	gravity	*g*
H	hour(s)	hr (in tables & parentheses)
I-J	indoleacetic acid	IAA
K	kilogram(s)	kg
L	least significant difference	LSD
	liter(s)	l (only in tabular matter because the abbreviation is easily confused with the figure "1")
M	maximum, maximal	max (in tables)
	meter	m
	microgram(s)	μg
	microliter(s)	μl
	micromolar(s) (unit of conc)	μM
	micromole(s) (unit of mass)	μmole
	milliequivalent(s)	meq
	milligram(s)	mg
	milliliter(s)	ml

(*Continued on next page*)

(*Continued from page* 878)

M cont. ...	millimicron(s)	mμ
	millimolar (unit of conc)	mM
	millimole(s) (unit of mass)	mmole
	minimum, minimal	min (in tables)
	minute(s)	min (in tables & parentheses only)
	molar (mole/liter)	M
	mole(s) (a gram molecule)	mole
N-O	normal (conc)	N
	normal (in trivial names in organic compounds)	*n*-
	number	No.
P	page	p (plural—pp)
	part(s) per million	ppm (*not* p. p. m.)
	per cent	% (with numeral & in tables)
	precipitate	ppt (in tables)
R	revolutions per minute	rpm
	ribonucleic acid	RNA
S	second(s)	sec (in tables only)
	species	sp. (when part of bionomial)
	square centimeter(s)	cm^2
	standard deviation	SD
	standard error	SE
T-U	temperature	temp (in tables)
	time measurements	(abbreviate in tables & parentheses only)
	triphosphopyridine nucleotide	TPN
V	variety(ies) (in taxonomy)	var. (when used with the binomial)
	versus	vs.
	volt(s)	v
	volume(s)	vol (in tables)
	volume/volume (conc)	v/v
W-Z	watt(s)	w
	weight	wt
	weight/volume (conc)	w/v

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

---

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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

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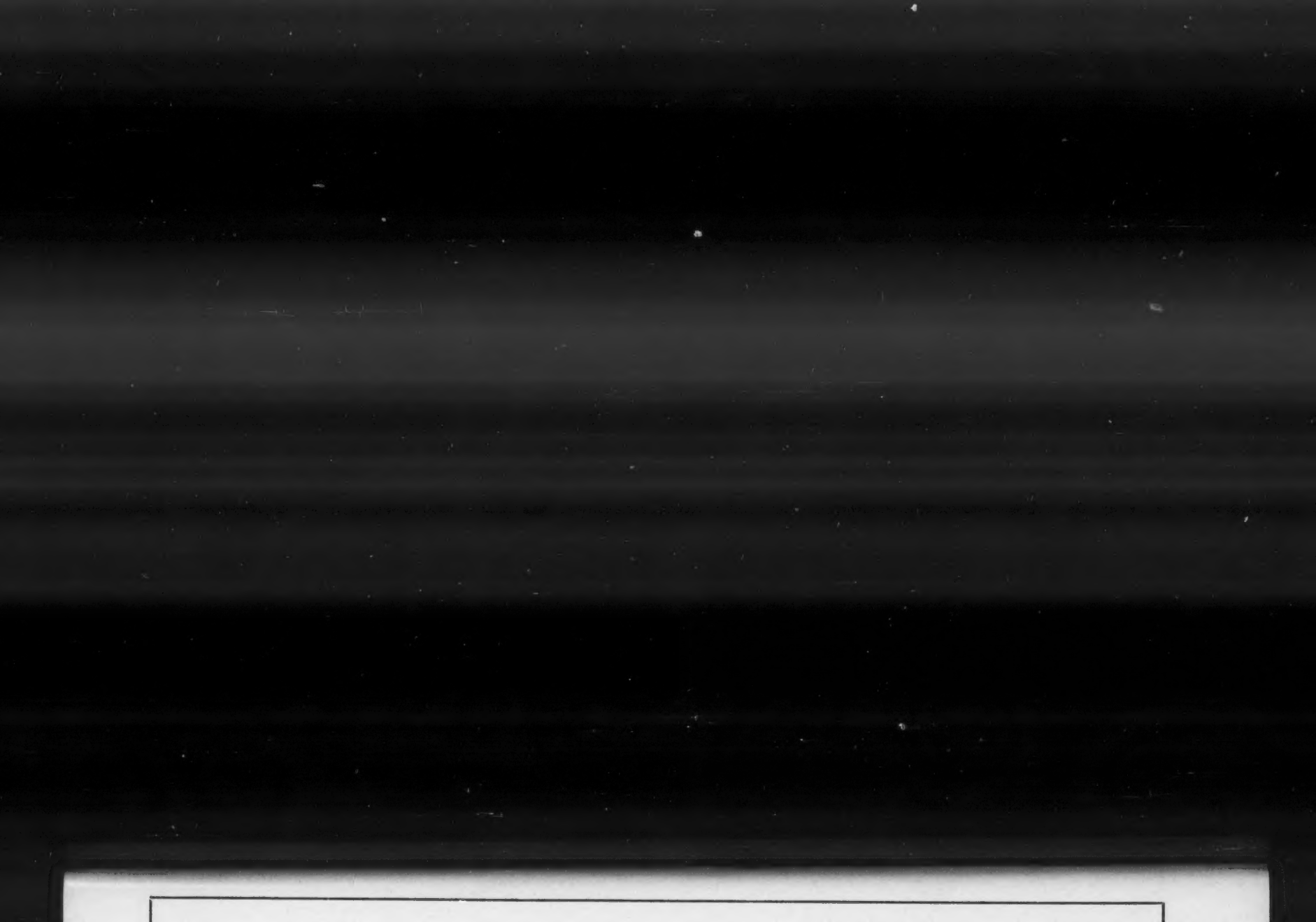


TABLE OF CONTENTS (Cont.)

Inhibitors of Hill Reaction. NORMAN E. GOOD 788

Oxidative Activity of Cytoplastic Particles of Apples: Krebs Cycle Oxidations, Oxidative Phosphorylation, & Cytochromes. MORRIS LIEBERMAN 804

Deoxyribonucleic Acid of Cotton. DAVID R. ERGLE & FRANK R. H. KATTERMAN 811

Physiological Effects & Degradation of S,S,S-Tributylphosphorotrithioate by Cotton Leaves as Shown by the P^{32} & S^{35} Labeled Compounds. FRANK R. H. KATTERMAN & WAYNE C. HALL 816

Carbohydrate Translocation in Raspberry & Soybean. J. WILLIAM A. BURLEY 820

Western X-Little Cherry Virus Effects on Respiration of Host Tree. R. O. BULKENCREN 826

Physiological Effects of Gibberellic Acid. III. *Observations on Its Mode of Action on Barley Endosperm.* LESLIE G. PALEG 829

Formation of Auxin From Tryptophan Through Action of Polyphenols. S. A. GORDON & L. G. PALEG 838

News & Notes 847

General Index, Volume 36 851

Table of Contents, Annual 867

Instructions for Contributors to Plant Physiology 873

Symbols & Abbreviations 877

TABLE OF CONTENTS

Translocated Heat Injury. C. E. YARWOOD 721

Growth & Geotropic Curvature of Avena Coleoptiles in Presence of n-1-naphthylphthalamic Acid. A. R. SCHRANK 727

Red, Far-Red Response & Chlorophyll Synthesis. LEONARD PRICE & WILLIAM H. KLEIN 733

Effect of Iron Chlorosis on Protein Fractions of Corn Leaf Tissue. N. G. PERUR, R. L. SMITH, & HERMAN H. WIEBE 736

Germination Inhibitors Related to Dormancy in Wheat Seeds. TAKAO MIYAMOTO, N. E. TOLBERT, & E. H. EVERSON 739

Identification & Mode of Action of a Component of Positively-Ionized Air Causing Enhanced Growth in Plants. RICHARD F. SMITH & WALLACE H. FULLER 747

An Inhibitor of Indoleacetic Acid Oxidase from Pea Tips. FRANKLIN E. MUMFORD, DEWEY H. SMITH, & JOHN E. CASTLE 752

Assimilation of C^{14}, N^{15} Labeled Urea by Excised Apple & Peach Leaves. DAVID R. DILLEY & DAVID R. WALKER 757

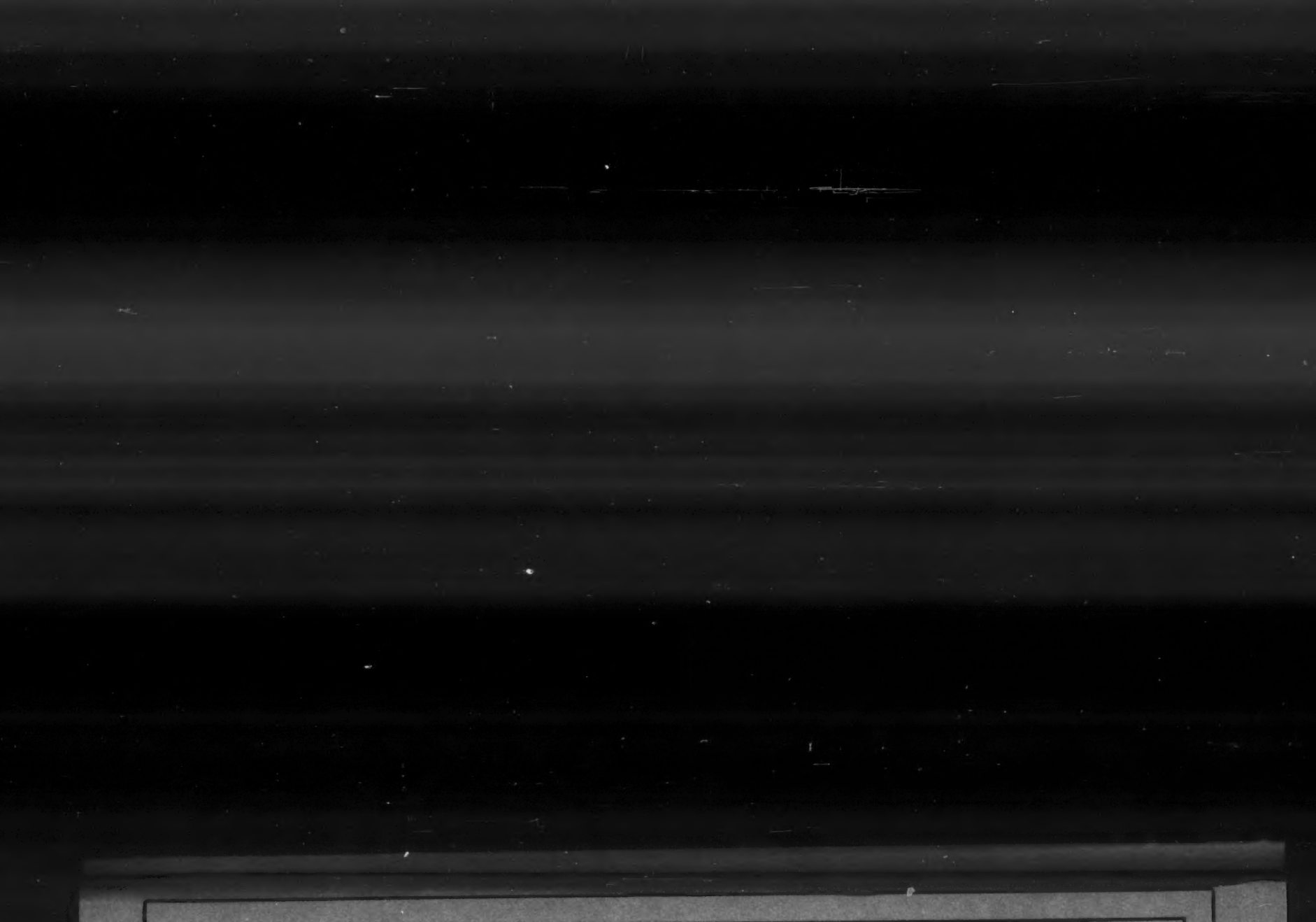
Effect of Soil Moisture Stress on Absorption & Translocation of Phosphorus Applied to Leaves of Sunflower. A. M. WILSON & CYRUS M. MCKELL 762

Uptake of Calcium & Chlorine in Roots of Zea mays. RAYMOND HANDLEY & ROY OVERSTREET 766

Changes in Nitrogen Metabolism in Sweet Potato With Black Rot. I. URITANI & MARK STAHMANN 770

Studies on 3-Indoleacetic Acid Metabolism. VII. *Metabolism of Radioactive 3-Indoleacetic Acid by Pea Roots.* W. A. ANDREAE, J. R. ROBINSON, & M. W. H. VAN YSSELSTEIN 783

CONTINUED ON INSIDE BACK COVER



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VOLUME 36 SUPPLEMENT

PLANT PHYSIOLOGY

PROCEEDINGS OF THE ANNUAL MEETINGS

PURDUE UNIVERSITY
LAFAYETTE, INDIANA

August 27-31, 1961

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THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

The American Society of Plant Physiologists

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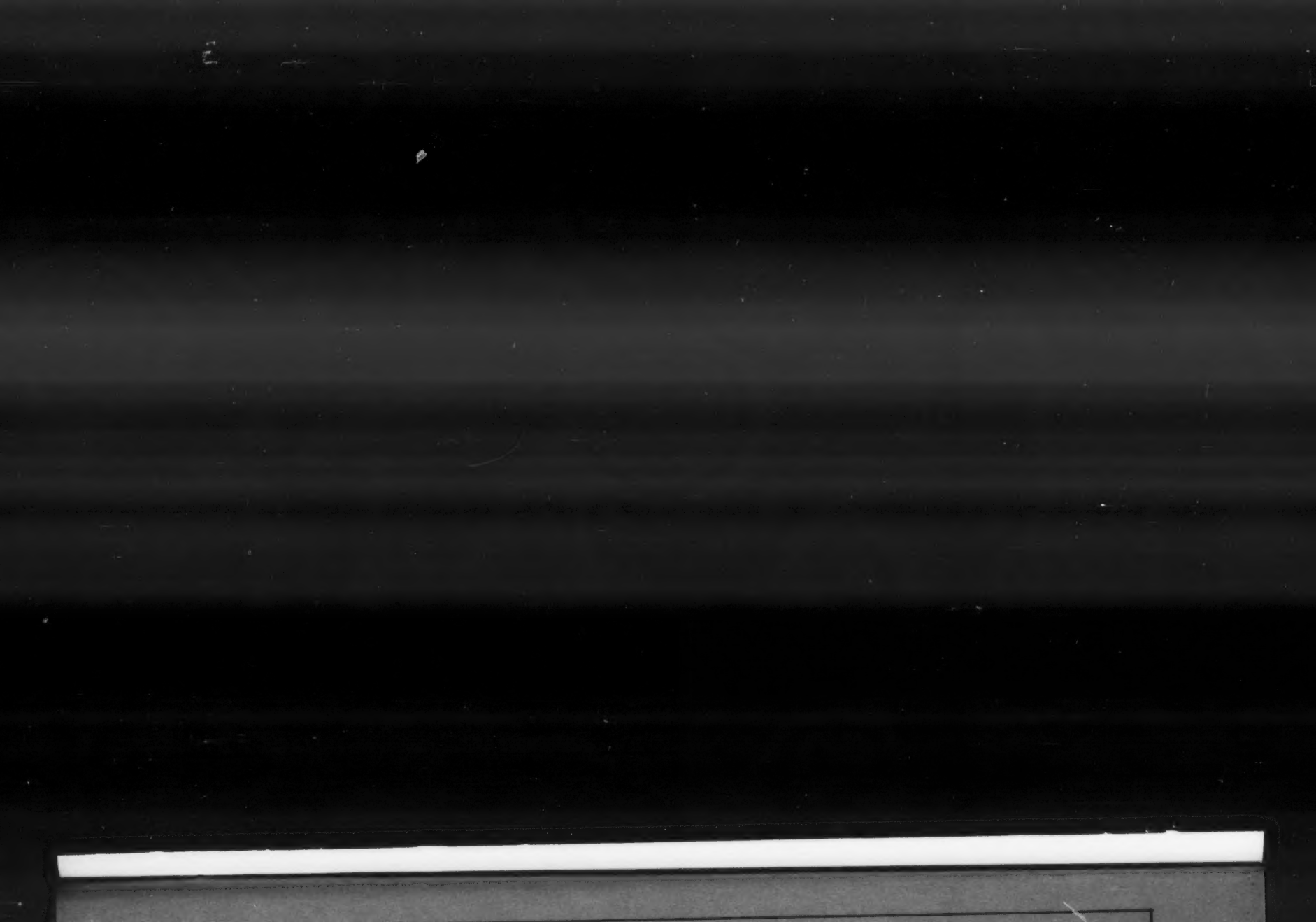


OUTLINE OF THE PROGRAM

	Monday, August 28	Tuesday, August 29	Wednesday, August 30	Thursday, August 31
8:25 A.M.	Announcements	Announcements	Announcements	Announcements
8:30 A.M.	A1 Photosynthesis I A2 Biochemistry I A3 Growth Regulators I A4 *Symposium* (9:00 A.M.): Weather and Organisms	C1 *Informal Discussion:* Chemotropism and Chemotaxis C2 Biochemistry II C3 Growth I C4 Water Relations	E1 Photosynthesis III E2 Growth II E3 Respiration E4 Translocation and Ion Uptake	G1 *Informal Discussion:* Metabolic Control Mechanisms G2 Biochemistry IV G3 Photosynthesis IV G4 Growth IV
	LUNCH	LUNCH	LUNCH	
1:30 P.M.	B1 Photosynthesis II B2 Growth Regulators II B3 Mineral Nutrition B4 *Symposium* (2:00 P.M.): Weather and Organisms	D1 *Symposium:* Frontiers in Plant Physiology D2 Business Meeting of The American Society of Plant Physiologists (to immediately follow symposium)	F1 *Informal Discussion:* Energy Transfer in Photosynthesis F2 Biochemistry III F3 Growth Regulators III F4 Growth III F5 Business Meeting of The Physiological Section of the Botanical Society of America (to immediately follow Session F4)	
6:30 P.M.	Annual Banquet of The American Society of Plant Physiologists			

PROCEEDINGS OF THE ANNUAL MEETINGS

WITH PAPERS FROM

THE
AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

AND THE

PHYSIOLOGICAL SECTION
BOTANICAL SOCIETY OF AMERICA

PURDUE UNIVERSITY
LAFAYETTE, INDIANA

August 27-31, 1961

The program for the meetings is outlined in the table on page i and the abstracts are presented in the pages which follow. Titles marked with an asterisk (*) are those submitted through the Physiological Section.

Please note that all contributed papers are scheduled for 15 minutes. *This limit will be strictly enforced by the chairmen of the various sessions.* A speaker desiring discussion after his talk should conclude his remarks early.

The number of papers to be given this year is quite large. This has caused many sessions to be unusually long. Furthermore, the program is running until Thursday noon. The program committee has scheduled what it considers *some of the best sessions for Thursday morning* in the hope that the members will remain until the end of our meetings. Persons presenting papers on Thursday deserve your attention.

The Editorial Board of Plant Physiology will meet at 7:30 on Sunday evening in Room 326 of the Memorial Center.

The Executive Committee will meet at 8:30 on Sunday evening in Room 326 of the Memorial Center.

Room 214 in the Union has been reserved for the ASPP for small meetings and will be available for small informal night sessions. Room 310 in the Memorial Center may be used for larger night sessions, if necessary.

Our annual banquet will start at 6:30 on Monday evening and will be held in the South Ballroom of the Union. The business meeting will take place Tuesday afternoon immediately following the conclusion of the Frontiers Symposium in Loeb Playhouse of the Memorial Center. The Physiological Section will have its business meeting immediately following Session F4 on Wednesday afternoon in Room 201 of the Civil Engineering Building.

The results of the 1961 election are tabulated at the bottom of the inside back cover of this *Supplement*. Approximately 710 members voted.

CARLOS MILLER, *Secretary*

MONDAY, AUGUST 28

MONDAY MORNING
CONCURRENT SESSIONS A1, A2, A3, A4

SESSION A1. Room 310, Memorial Center

PHOTOSYNTHESIS I

A. T. Jagendorf, *presiding*

8:25. Announcements.

8:30. BROWN, T. E., Charles F. Kettering Foundation, Yellow Springs, Ohio. Physiological aspects of the growth of normal and manganese-deficient *Chlorella* in the presence of various atmospheres.

These studies were made in line with our continuing efforts toward clarifying the effect of Mn-deficiency and the associated connection with glycolic acid.

Various percentages and combinations of oxygen, carbon dioxide, nitrogen, argon and helium were used as growth atmospheres for normal and Mn-def. *Chlorella pyrenoidosa.* Along with the monitoring of growth, chlorophyll, quinone Hill reaction, photosynthesis, respiration, and glycolic acid excretion determinations were made.

An additional study of inoculum size has shown that the growth of Mn-def. cells under N_2 is greatly dependent upon this factor. It is also shown that Mn-def. cells are strongly hampered by high oxygen concentrations which are not effective with normal cells.

8:45. EYSTER, CLYDE, Charles F. Kettering Foundation, Yellow Springs, Ohio. Relation of manganese to photosynthetic reduction of TPN.

A method has been developed which will extract photosynthetic pyridine nucleotide reductase, PPNR, from whole cells of *Chlorella pyrenoidosa.* PPNR from normal manganese-sufficient Chlorella and PPNR from manganese-deficient Chlorella were compared as to their abilities to reduce TPN in reaction systems using spinach chloroplast grana. Both were quite active but the greater activity was always associated with the plus Mn grown cells. Active grana have been prepared from normal Chlorella and these have been used to determine whether Mn is a necessary constituent for active PPNR. Grana from Mn-deficient Chlorella did not seem to be able to reduce TPN regardless of whether PPNR from plus Mn Chlorella or PPNR from minus Mn Chlorella was added. It is tentatively concluded that, for grana to be active in the reduction of TPN, manganese is required.

9:00. BANNISTER, T. T. and M. J. VROOMAN, Univ. of Rochester, Rochester 20, N.Y. Absence of enhancement in the 2,6-dichlorophenol Hill reaction of oats.

In Chlorella, short waves ($\lesssim$ 670 mμ) enhance the rate of oxygen-evolution in far red light (695 mμ), both of whole-cell photosynthesis and of the Hill reaction with quinone. Moreover, unequal rates result with saturating illuminations of short-wave and far red light. These findings have been interpreted to mean that two different photochemical reactions are involved in photosynthesis.

In the 2,6-dichlorophenol-indophenol Hill reaction of isolated oat chloroplasts, neither enhancement nor unequal saturating rates could be demonstrated. In our experiments, "enhanceable" light (695 mμ) was obtained with an interference filter, and enhancing light consisted of white or blue (470 mμ) light. With low illuminations, the rates of reduction of DCIP (measured spectrophotometrically) were additive; with saturating illuminations the rates were equal. These results suggest that only one of the postulated photochemical reactions is involved in the DCIP Hill reaction.

9:15. STERN, BABETTE, Univ. of Chicago, Chicago, Ill. The carbon dioxide requirement for the Hill reaction.

Warburg and Krippahl (*Z. Naturforsch.*, 15 b, 367, 1960) have previously proved that CO_2 is required for the photoevolution of O_2 by Chlorella and kohlrabi grana. In a preliminary communication, (*J. Biol. Chem.*, 235, PC51, 1960) we have verified this conclusion, using a system in which spinach or kohlrabi grana photoreduce ferricyanide in the presence of catalytic amounts of the dye, trichlorophenol indophenol. Additional evidence will be presented which indicates that the CO_2 requirement is a general phenomenon associated with the photoevolution of O_2 by spinach chloroplasts when ferricyanide, various indophenol dyes, TPN and FMN function as Hill oxidants. A more detailed study of the factors influencing the CO_2 requirement for ferricyanide reduction will also be presented.

With our experimental conditions, the elimination of CO_2 from the reaction mixture, in the presence of alkali, is a function of the time of incubation in the dark, the length of the illumination period, the chlorophyll concentration, the buffer employed, the particular spinach preparation, and the pH of the reaction mixture.

The rate of ferricyanide reduction is stimulated by the presence of higher concentrations of CO_2 and HCO_3^- than are normally found in solutions in equilibrium with room air at pH 6.8. If trichlorophenol indophenol or NH_4^+ are present during the photoreduction of ferricyanide, the magnitude of the CO_2 effect is enhanced, because these substances accelerate the rate of ferricyanide reduction relatively more in the presence of CO_2 than in its absence.

9:30. FREDERICKS, W. W. and A. T. JAGENDORF, Johns Hopkins Univ., Baltimore, Md. Restoration of Hill activity in washed chromatophores of *Anacystis.*

B. Petrack observed (*Fed. Proc.* 1959) that cell-free extracts of the Blue-green Alga, Anabaena, isolated in Carbowax exhibited two photochemical activities: a) the "Hill Reaction" and b) Cyclic Phosphorylation. Further she observed that on washing the chromatophores with water; a) Cyclic Phosphorylation was retained, b) the Hill Reaction was lost and c) the aqueous extract now contained the accessory pigment, phycocyanin.

A similar system has been developed in Anacystis, showing the same phenomenon with respect to the Hill Reaction. Pre-

liminary experiments indicate that it is possible to reconstitute the system and restore the Hill activity. The characteristics of the reconstruction process and the nature of the active component(s) in the extract will be discussed.

9:45. BANNISTER, T. T., Univ. of Rochester. Evidence against the reported reduction of DPN sensitized by chlorophyll in solution.

Krasnovsky and Brin (*Doklady Akad. Nauk* SSSR 67: 325, 1949; *Bioximiya* SSSR 24: 1085, 1959) reported that, in aqueous pyridine, chlorophyll *a* sensitized the reduction of DPN+ by ascorbic acid. As evidence, they claimed that (1) DPN+ reoxidized photoreduced chlorophyll, (2) the absorption peak of DPNH at 340mμ appeared when a solution of chlorophyll, DPN+, and ascorbic acid was illuminated with red light, and (3) DPNH was isolated from illuminated solutions. These findings now appear doubtful.

In preliminary experiments, DPN+ reduction could not be detected by either fluorimetry or spectrophotometry, but complex changes in UV absorption were observed to which DPN+ reduction, in small amounts, could have contributed. Carefully controlled experiments have led to identification of some of the processes contributing to the UV changes. The results, although still not completely ruling out DPN+ reduction, prove by their complexity that Krasnovsky and Brin interpreted their observation too simply.

10:00. Recess.

10:15. VAN NORMAN, R. W., R. B. SHAFER and B. J. REE, Univ. of Utah, Salt Lake City, Utah. Effects of X-rays on solutions of chlorophylls.

Acetone solutions of chlorophyll a, chlorophyll b and prepared mixtures of the two were subjected to X-rays in doses up to 31 600 r. Destruction of pigments was followed by measuring changes in absorption spectra. Disappearance of pigments is linear with radiation dosage, but chlorophyll a is more sensitive than chlorophyll b. The red absorption band of chlorophyll a falls more rapidly than the blue asborption band; chlorophyll b shows a similar but smaller effect. Variations in water content of the acetone produce only small differences. The data confirm and extend our earlier results, even though they are directly contradictory to some recent Japanese work. Reasons for the discrepancies are discussed.

10:30. BUTLER, WARREN L., Plant Industry Station, Beltsville, Md. Fluorescence excitation spectra of leaves during the induction period of photosynthesis.

Fluorescence excitation spectra of leaves have been employed to study the fluorescence anomalies that occur during the induction period of photosynthesis. The excitation spectra show that the fluorescence of wavelengths longer than 730 mμ is due to emission from chlorophyll a and C-705. If the leaf is irradiated with a moderately strong red source the well known transient increase of fluorescence occurs. Fluorescence excitation spectra were measured immediately after a brief irradiation with red light. The increased level of fluorescence resulting from the brief illumination persists for several minutes in air and much longer in N_2. The excitation spectra show that the transient increase of fluorescence is due to emission from chlorophyll a but not C-705.

It will be shown that the increased fluorescence yield of chlorophyll a resulting from red light can be counteracted by irradiation with far-red light. The fluorescence yield of chlorophyll a can be alternately increased with red light and decreased with far-red in a series of brief irradiations.

The changes in fluorescence yield will be discussed in terms of the quenching of chlorophyll a fluorescence by C-705 acting as an energy sink.

10:45. LIPPINCOTT, JAMES A., JACQUES AGHION and WALTER F. BERTSCH, Northwestern Univ., Evanston, Ill. The appearance of a 740 mμ absorption band on treatment of leaves and isolated chloroplasts with organic solvents.

When chlorophyll-containing leaves are homogenized in appropriate concentrations of methanol, ethanol, acteone, n-propanol or t-butanol, most of the absorbancy at 660-680 mμ disappears and a new absorbancy maximum at about 740 mμ is observed. Similar results may be obtained using isolated chloroplasts. Factors affecting this change will be described. The material absorbing at 740 mμ is particulate and can be demonstrated to be associated with chloroplast particles by microscopic examination. Phytol is still present in the chlorophyll extracted from these preparations. The fluorescent and spectral properties of this material are such as to suggest that it represents a crystalline form of chlorophyll associated with chloroplast fragments. By temperature changes at appropriate solvent concentrations the absorbancy of these preparations may be shifted reversibly from 740 mμ to 660 mμ. No evidence was obtained to associate such spectral changes with the red, far-red photomorphogenic pigments, nor was this spectral shift influenced by light.

11:00. LOVE, BRUCE B., Univ. of Rochester, Rochester, N.Y. Studies of a chlorophyll-dioxane complex.

Preparations of chlorophyll *a* in aqueous dioxane have well defined absorption spectra, with a maximum at 682 mμ in the red region. At dioxane concentrations below 0.05 M the chlorophyll is almost completely in the 670 mμ colloidal state. At concentrations above 0.6 M dioxane is almost completely converted to the 682 mμ colloidal state. At intermediate dioxane concentrations mixtures of the two colloidal states coexist. The kinetics of the conversion of the 670 mμ colloid to the 682 mμ colloid, as a function of dioxane concentration, has been studied. The rate law established was used to predict the mixtures obtained at intermediate dioxane concentrations.

Ethyl chlorophyllide dioxane complexes, but not pheophorbide, can be produced. This indicates that the central metal atom of the porphyrin is the locus of interaction with dioxane. This is further supported by the fact that Zn pheophorbide, but not Cu or Co pheophorbides, can form a dioxane complex. The spectra of the chlorophyll-dioxane complex and chlorophyllide-dioxane complex are identical. This result shows that the phytol side chain is not necessary for complex formation. Comparison of the rates of formation of complex with chlorophyll and chlorophyllide indicates that the phytol side chain may offer considerable steric hindrance to complex formation. The chlorophyllide-dioxane complex undergoes a slow decay into chlorophyllide microcrystals, at a rate which is independent of dioxane concentration.

11:15. KLEIN, ATTILA O. and WOLF VISHNIAC, Yale Univ., New Haven 11, Conn. A chlorophyllase preparation and its assay in aqueous media.

An active chlorophyllase preparation was obtained from 7 day-old etiolated rye seedlings. The particulate fraction of the homogenate was treated with 10% iso-butanol and extracted with 1% sodium desoxycholate (SDC). A precipitate obtained by making the SDC fraction 30% saturated with ammonium sulfate was extracted with phosphate buffer. The chlorophyllase activity of this last extract represented a 500-fold purification over the chloroplast fraction. The enzyme is still considered to be an SDC-chlorophyllase complex of particulate nature.

The aqueous assay system contained pheophytin *a* as substrate which was solubilized by the presence of 0.2% Trition X-100 in the phosphate buffer. After incubation the pheophytin *a* was partitioned between acetone and petroleum ether the optical densities of which yielded the amount of hydrolyzed and unhydrolyzed substrate respectively.

The equilibrium point of 85% hydrolysis was approached after 10 hours of incubation. The K_M for pheophytin *a* was 1.5×10^{-5} and the reaction exhibited first order kinetics with respect to enzyme. Inhibitor studies indicated the presence of sulfhydryl groups. The localization and functions of chlorophyllase in the chloroplast will be discussed.

SESSION A2. Room 318, Memorial Center

BIOCHEMISTRY I

E. R. Waygood, *presiding*

8:25. Announcements.

8:30. BEREZNIAK, H. and DALTON WANG, Univ. of Manitoba, Winnipeg, Canada. The metabolism of 5-amino-4-imidazolecarboxamide-2-C^{14} in wheat embryos.

Wheat embryos which had been incubated with 5-amino-4-imidazolecarboxamide-2-C^{14} were fractionated into acid soluble, ribonucleic acid, and deoxyribonucleic acid fractions. The radio-active compounds in each fraction were analyzed. Results obtained showed that 5-amino-4-imidazolecarboxamide-2-C^{14} were efficiently utilized by the wheat embryo as an intermediate in the biosynthesis of purines and their derivatives. The specific activity of these compounds was determined and its significance will be discussed.

8:45. KAPOOR, M. and E. R. WAYGOOD, Univ. of Manitoba, Winnipeg, Canada. Enzymes of orotic acid metabolism in wheat embryos.

Orotidine-5-phosphate pyrophosphorylase and dihydroorotic dehydrogenase have been isolated from wheat embryos. Kinetic studies have been conducted on both the enzymes. Orotidine-5-phosphate pyrophosphorylase of wheat embryos has also been used for the assay of 5-phosphoribose-1-pyrophosphate by the method of Kornberg, Lieberman and Simms (1955). The results obtained will be discussed.

9:00. KING, J. and DALTON WANG, Univ. of Manitoba, Winnipeg, Canada. Biosynthesis of pyrimidines and their derivatives in the wheat plant.

Detached leaves or embryos of Selkirk wheat were incubated with aspartic acid-2-^{14}C or uracil-2-^{14}C. Alcohol or cold perchloric acid, dilute alkali, and hot perchloric acid fractions were obtained in which a number of labelled pyrimidine were identified. The specific activities of these compounds have been determined and the significance of the results will be discussed.

9:15. BEREZNIAK, N. and DALTON WANG, Univ. of Manitoba, Winnipeg, Canada. The study of free nucleotides in wheat embryos.

Wheat embryos were incubated with inorganic phosphate labelled P^{32}, and the cold acid soluble nucleotides were extracted and analyzed by means of selective adsorption, paper chromatography, and radioautography. At least 21 uv absorbing compounds were labelled. Those compounds containing P^{32} were identified as nucleotides by means of co-chromatography and analysis of their hydrolytic products.

9:30. PLAISTED, PHILIP H. and R. REGGIO, Boyce Thompson Institute for Plant Research, Inc., Yonkers, N.Y. A twenty-four hour separation of acid soluble nucleotides.

The separation of acid soluble nucleotides requires considerable time. Initially, the compounds are extracted from plant tissue, then the extract is semipurified by adsorption and elution from charcoal, followed by liquid-liquid extraction with ethyl ether. Probably the greatest resolution of the semipurified extract is obtained by eluting the nucleotides from a column of anion resins. This elution step requires from seven to twelve days, depending upon the system used. Recently this laboratory has developed an elution system that shortens the time to 24 hours.

The nucleotide extract is eluted from a Dowex 1 (formate) column by an increasing concentration of formate ions. If the concentration of formic ions passing through the column is plotted against the volume of liquid which has passed through, an S-shaped curve is generated. This type of curve is obtained by using a five or nine chambered reservoir to hold the eluting liquids. Each reservoir is filled with different concentrations of formic acid-sodium formate. The shape of the elution curve can be changed by altering the concentration in any one container. Graphs showing resolution of authentic samples and nucleotides from plant tissue will be shown.

9:45. STERN, H. and Y. HOTTA, Univ. of Illinois, Urbana, Ill. Properties of polymerized DNA from tissues of higher plants.

The preparation and properties of DNA obtained from a number of higher plants will be discussed. Data will be presented covering the intactness of the molecules isolated. Differences in DNA arising through differentiation will be considered.

10:00. Recess.

10:15. CORRELL, D. L., Univ. of California, La Jolla, Calif. Properties of RNA-polyphosphates isolated from *Chlorella pyrenoidosa.*

RNA-polyphosphates were isolated from synchronous 20 liter cultures of *Chlorella pyrenoidosa* (V.N. Z 2.2.1). The isolation was initiated by thawing the harvested and frozen cells in distilled water which was adjusted at intervals to

pH 11.5 with KOH. After one hour at room temperature the pH was adjusted to 7.4 and two phenol extractions were carried out. The residual phenol was removed with ether. One percent solid sodium acetate and four volumes of alcohol were then added and more sodium acteate was added with stirring until a flocculent precipitate appeared. The solution was then put in a deep freeze for one hour. The precipitate was washed with alcohol, dried at reduced pressure, and chromatographed on a DEAE-cellulose column at pH 7.6 using a sodium chloride gradient.

During a nine hour period prior to nuclear division in the *Chlorella* a distinctive type of RNA-polyphosphate was isolated. The metachromatic reaction of toluidine blue with this RNA-polyphosphate was small. However, after 24 hours dialysis against distilled water, a large metachromatic reaction was observed. This change was not induced by dialysis against salt concentrations above 0.2 M between pH's 6 and 8. Yeast polyphosphatase was also more effective after dialysis. Mild alkaline hydrolysis greatly reduced the metachromasy without hydrolyzing the polyphosphate. These and other data suggest that the RNA and polyphosphate were linked by covalent bonds and also, perhaps, by hydrogen bonds, which could be disrupted by dialysis against distilled water.

10:30. CHERRY, J. H. and R. H. HAGEMAN, Univ. of Illinois, Urbana, Ill. Effects of X-irradiation of corn seed on RNA metabolism.

The irradiation of corn seed with X-rays drastically retards germination and seedling growth. Data presented at the 1960 meeting showed that changes in activities of certain glycolytic enzymes and mitochondria can not account for the impairment of growth by X-irradiation.

In the meristematic region of both root tips and mesocotyls, X-irradiation affected a distribution of RNA among the cytoplasmic particulates much the same as expansive growth (Lund, *et. al. Biophys. Biochem. Cytol* 4:87, 1958). Subsequent studies indicate that growth following X-irradiation is largely by cell expansion. Differential ultra-centrifugation and RNA analysis show that microsomal RNA of X-irradiated radicals disappear with growth. Concomitantly with the disappearance of microsomal RNA, there is an increase in mitochondrial RNA.

The incorporation of precursors into the nucleic acids of the subcellular components of intact seedlings and excised root tips shows that the nuclear material incorporates more radioactivity, followed in order by the mitochondrial, soluble and microsomal RNA fractions.

Evidence is presented for a relation between RNA metabolism, cell maturation and nuclear function as a fundamental part of plant growth.

10:45. CHERRY, J. H. and R. H. HAGEMAN, Univ. of Illinois, Urbana, Ill. Effects of X-irradiation of corn seed on RNase activity.

Evidence presented in the previous paper indicates that microsomal RNA is metabolized during cell expansion and that X-irradiation affects this metabolism.

Since X-irradiation inhibits cell division, but apparently has little or no effect on cell expansion, growth from X-irradiated seed is primarily by cell elongation.

Subsequent studies indicate that exposure of corn seed to X-irradiation greatly enhances the activity of RNase, especially in the root-tip meristem. Actively dividing cells are characterized by a low RNase activity. RNase activity increases with cell growth and maturation.

These results suggests that RNase is involved in the metabolism and transfer of RNA, especially microsomal, during expansive growth.

11:00. WILSON, C. M., U. S. Dept. of Agriculture, Univ. of Illinois, Urbana, Ill. Purification and properties of ribonucleases in corn seedlings.

Two ribonucleases in corn root tissue have been separated and partially purified. The ribonucleases were adsorbed on a column of carboxymethyl cellulose equilibrated with pH 5.0 citrate buffer while most of the soluble proteins were not adsorbed. One ribonuclease was eluted with pH 6.0 citrate buffer, whereas the second was subsequently eluted with pH 8.0 tris buffer containing 0.1 M NaCl. The first enzyme has a pH optimum near 5.4 and the second near 6.2. The effect of salts was investigated by using a cacodylic acid-imidazole buffer containing no metallic cations. The first enzyme showed increasing activity up to 0.1 M KCl and the second up to 0.2 M KCl and also up to 0.04 M $MgCl_2$. The second enzyme was inhibited by higher concentrations of $MgCl_2$ while the first was inhibited by low concentrations. The ribonuclease with the high pH optimum is found in the particulate fraction of the cell.

Isolated scutella from germinating seedlings secreted up to half of their total ribonuclease into water in 2 hours. The secreted ribonuclease resembled the first enzyme described in its elution from a column and in its pH optimum.

11:15. SANTILLI, VINCENT and NICK C. GAGLIARDI, Univ. of Buffalo, Buffalo 14, N.Y. The hypochromicity induced by pancreatic ribonuclease (PRNase) in its initial reaction with yeast and tobacco mosaic virus (TMV) ribonucleic acid (RNA).*

When PRNase is added to yeast or TMV RNA (20-25 μg/ml) to give a final concentration of 10^{-3}-10^{-8} μg/ml, the optical density of the mixture at a wavelength of 260 mμ immediately drops by as much as 3%. After the initial drop the optical density gradually rises as the RNA is hydrolyzed. Hypochromicity is followed by a lag period during which no change in the optical density takes place. The lower the enzyme concentration the longer the lag period. At higher enzyme concentrations the hypochromic effect is masked by the hyperchromic effect. Hypochromicity has been produced by PRNase from two different sources and with RNA in acetate, phosphate and versene buffers at pH 7. RNA from two strains of TMV as well as commercial yeast RNA was susceptible to this effect. Dialyzing the enzyme does not and heating does remove its ability to produce hypochromicity. This effect cannot be accounted for by a shift in the spectrum. Both $MgCl_2$ and protamine sulfate at molar concentrations considerably greater than the enzyme produce hypochromicity. The addition

of either of these substances to RNA decreases the degree of hypochromicity subsequently produced by the enzyme, as well as the rate of its hydrolytic activity. When the initial velocity of hydrolysis by PRNase is plotted against enzyme concentration, it is found that the velocity at low concentrations is less than one would expect from the dilution. A theory involving an initial contraction of the substrate by the enzyme, which in turn causes an inhibition of hydrolytic activity, is put forward as a possible explanation for these results.

11:30. SANTILLI, VINCENT, CARL M. NEPOKROEFF and NICK C. GAGLIARDI, Univ. of Buffalo, Buffalo 14, N.Y. Agar plate assay for leaf ribonuclease in healthy and tobacco mosaic virus infected leaves.*

Fifteen ml of 2% agar and 0.125% yeast ribonucleic acid in 0.05 M sodium citrate buffer at pH 5.5 were allowed to solidify in a standard petri dish. Four equidistant wells 9 mm in diameter were cut out and filled with extract and the dish incubated at 37°C for 24 hours. At the end of incubation the remaining non-hydrolyzed ribonucleic acid in the dish was precipitated by the addition of 0.75% uranium acetate in 25% perchloric acid resulting in an opaque background surrounding the cleared zones around the wells where the enzyme from the extract had digested the nucleic acid. After 10 minutes the cleared zone diameters were measured. For pancreatic ribonuclease best results were obtained if the agar was buffered at pH 7 with 0.05 M NaH_2PO_4. When the diameters of the cleared zones were plotted against the log of the enzyme concentration (0.01-1.0 μg/ml) a straight line was obtained from which equivalents of pancreatic ribonuclease present in the extracts could be determined. Young systemically infected *Nicotiana sylvestris* leaves 13 days after the plants had been inoculated were found to have at least twice the ribonuclease content of comparable healthy leaves on a per unit area or per unit weight of protein basis. *Nicotiana glutinosa* half leaves, 40 hours after inoculation but before lesion appearance, were found to have 16% and 31% more ribonuclease per unit area and per unit weight of protein, respectively, than the opposite, healthy, buffer-inoculated half leaves.

SESSION A3. Room 4, Memorial Center

GROWTH REGULATORS I

F. G. Teubner, *presiding*

8.25. Announcements.

8:30. RITZERT, R. W., H. M. SELL and M. J. BUKOVAC, Michigan State Univ., East Lansing, Mich. Synthesis and biological activity of several ethyl 1-acylindole-3-acetates.

Synthesized derivatives of indole-3-acetic acid (IAA), with the exception of ethyl indole-3-acetate (IAE), are generally equal to or less active than the parent compound. IAE is an exception in that greater activity than IAA has been demonstrated in several physiological systems. Several ethyl 1-acylindole-3-acetates were synthesized and the effects of substitution on biological activity was determined. Equilmolecular amounts of IAE and the appropriate acyl chloride were refluxed in benzene or toluene for 24 to 48 hours. The following acyl groups were substituted in this manner: chloroacetyl, dichloroacetyl, 2-chloropropionyl, 3-chloropropionyl, and 4-nitrobenzoyl. The 4-aminobenzoyl derivative was prepared by reduction of 4-nitrobenzoyl IAE. Acetyl IAE was obtained by condensation of 1-acetylindole and ethyl diazoacetate. Comparative biological activity was determined in the promotion of parthenocarpic tomato ovary growth, bean petiole abscission, and *Avena* coleoptile straight growth. In the promotion of parthenocarpic growth of tomato ovaries, at 10^{-3} M, dichloroacetyl IAE was as active as IAE, and both were more active than IAA. Acetyl IAE and chloroacetyl IAE were less active than IAE but equal to IAA. The 2- and 3-chloropropionyl, 4-nitrobenzoyl, and 4-aminobenzoyl IAE did not promote parthenocarpic growth of tomato ovaries. Dichloroacetyl IAE was the most active compound in delaying bean petiole abscission. Acetyl, chloroacetyl, and 4-nitrobenzoyl IAE were slightly more active than either IAE or IAA. However, 2- and 3-chloropropionyl, and 4-aminobenzoyl IAE did not significantly delay petiole abscission. All of the IAE derivatives synthesized were active in the *Avena* coleoptile straight growth test over a wide concentration range. There was no marked effect due to substitution on the 1-indole position on biological activity of IAE as measured by the *Avena* straight growth assay.

8:45. POWELL, LLOYD E., Cornell Univ., Ithaca, N.Y. Solvent systems for silica gel column chromatography of indole derivatives.

An earlier paper (*Plant Physiol.* 35: 256-261. 1960.) described a method for separating a number of indole derivatives, employing silica gel partition columns and petroleum ether: n-butyl alcohol eluting solvents. This research has been extended so that the chromatographic behavior of several indole derivatives, i.e. ethyl indoleacetate, indoleacetonitrile, indolebutyric acid, indoleacetic acid, indoleacetamide, tryptophan, and tryptamine, is now known when any one of about 20 eluting solvent systems is employed. Most of these solvent systems use n-hexane as one component, although n-pentane and petroleum ether (b. p. 30-60°C.) have been used in a few of them. Alcohols, esters, chlorinated hydrocarbons, and a ketone (2-butanone) were studied as the second component.

As a group the lower boiling aliphatic alcohols capable of being saturated with water appeared to have the most value, especially sec-butyl, isobutyl, and t-amyl alcohols. A number of esters such as ethyl formate, and methyl and ethyl acetates were also useful, although they could not elute a given indole derivative as readily as the alcohols. The esters have the advantage of lower boiling points. 2-Butanone was similar to the esters. The chlorinated hydrocarbons were least satisfactory as a group, but of value for some derivatives.

Later investigations indicated that n-pentane was better than n-hexane for obtaining separation of ethyl indoleacetate and indoleacetonitrile when used with the alcohols. Petroleum ether gave results more nearly like those of pentane.

9:00. BLUMENTHAL-GOLDSCHMIDT, S., F. HAYASHI and LAWRENCE RAPPAPORT, Univ. of California, Davis, Calif. Extraction, separation, and detection of certain natural growth substances in potato tubers.

Accumulating evidence suggests that the rest period may be regulated by many growth substances possibly including

auxins, gibberellins, and certain growth inhibitors. The interpretation of their roles in relation to rest depends in part upon their separation, detection and ultimately, identification. The same sample of potato peelings was successively extracted with several solvent systems and the extracts were exhaustively separated by liquid: liquid partitioning, and column and paper chromatography. Chromatograms were inspected with ultraviolet light and by color detection. Activity was tested on dwarf pea, dwarf corn, Avena first internode, wheat coleoptile and potato eye bioassays. No single solvent system extracted complete activity of a class of compounds—auxins, gibberellins, or inhibitors. Succeeding solvent extractions usually provided additional growth substance activity, both inhibiting and stimulating. The explanation of these results and their significance in interpreting the role of growth substances in the rest period will be discussed.

9:15. RAPPAPORT, L. and S. BLUMENTHAL-GOLDSCHMIDT, Univ. of California, Davis, Calif. A potato "eye" bioassay for growth substances.

Critical interpretation of the role of extracted growth substances in the rest period depends in part upon the use of appropriate bioassays. With few exceptions, the oat internode or wheat coleoptile are used to measure both growth stimulation and inhibition. The results are related to the events leading to emergence from rest. Too often, however, as with potato extracts, growth substances found to inhibit coleoptiles fail to prolong rest in either intact potatoes or in sections containing buds. Since no specific bioassay for inhibitors is available it is desirable to test activity on tissues of the same species that is extracted. Therefore, the effect of various growth substances was studied on sprouting of buds on cylinders of potato tuber about 1.0 cm long, removed with a No. 3 cork borer. The cylinders are placed on Whatman No. 1 filter paper treated with water or solutions containing growth substances, or measured droplets are applied directly to the eye. Application of gibberellins, indole-3-acetic acid, coumarin and several other growth substances directly on the eye affected the rate of sprouting more than applying them to the filter paper. Exceedingly low doses of gibberellin A_1 and A_3 (total: less than 10^{-4} μg per cylinder) were effective in stimulating sprouting, whereas repeated treatment on the bud with comparatively high concentrations of coumarin did not affect the time to initial sprouting, but increased the time for 50% of the population of treated buds to sprout. The significance of these results in studying the balance concept of growth promotion and inhibition will be discussed.

9:30. GUPTA, P. K., R. W. CAMPBELL and P. K. YU, Kansas State Univ., Manhattan, Kan. Occurrence of indigenous auxins in strawberry seeds.

The auxin concentrations in the achenes located on the basal, median and distal portions of Armore, Pocahontas and Surecrop berries were measured. A portion of the study was devoted to determining the correlation between ease of germination and location of achenes on the berry. Paper chromatography was employed to separate growth substances and the *Avena* first-internode growth test was used. Identification of auxins was made on the basis of Rf values and also by the use of ultra violet light as well as Ehrlichs reagent.

The auxin concentration in the achenes from median and distal portions of the berries Armore and Pocahontas varieties was found to be significantly higher than in achenes from basal portions. The achenes collected from the basal portion of Surecrop berries had a significantly higher auxin concentration than achenes located on the median and distal portions. No significant differences have been observed between the auxin concentration in the achenes of the three varieties when whole berries were compared. A gradual decrease in auxin content of the achenes resulted from refrigerated storage for one year or longer. These results will be discussed in the light of the differences observed in the germination of seeds.

9:45. YU, P. K., R. W. CAMPBELL and P. K. GUPTA, Kansas State Univ., Manhattan, Kan. Growth substances in developing peach fruit.

These investigations were undertaken to determine the correlation between the typical signmoid growth curve of the peach fruit and occurrence of indigenous growth substances. Seasonal trends in the level of growth substances of the fleshy pericarp and seed portion of three different varieties were established. Fruit samples were collected at weekly intervals from petal fall until maturity. Auxins were extracted with peroxide-free ether from ground lyophilized samples. Crude auxins were purified by use of a mixture of hexane and acetonitrile preceding separation by paper chromatography. Bioassay was by the *Avena* first-internode straight growth test.

Results indicated the growing peach fruit produced four to five different substances which are capable of inducing growth of the oat seedling internode. The differences between induced growth results from the different substances, however, were nonsignificant. The seasonal differences and the difference between amounts of growth substances found in flesh and seed portions were highly significant in all three varieties. The relatively higher amount of auxins in the fleshy pericarp during the period of rapid fruit growth suggests the growth substances, found on the different positions of the paper chromatogram to be synthesized in the seed and transported to where growth occurs.

10:00. Recess.

10:15. SAHAI, B. I. and MICHAEL SHAW, Univ. of Saskatchewan, Saskatoon, Canada. Growth substances in coconut meat and wheat leaves.

Coconut meat and wheat leaves were extracted with cold ethanol. Ether soluble substances were removed and the aqueous fraction was then treated with cuprous oxide to precipitate purines. The copper was removed by treatment with H_2S and the purines were separated by chromatography on Dowex I resin and paper chromatography in n-Butanol: Acetic acid: water (4:1:5). Adenine and adenosine were isolated in crystalline form from coconut meat and adenine was isolated from wheat leaves. The identity of these substances was confirmed by ultraviolet and infra red spectrophotometry. Several other unidentified purine-like substances with characteristic ultraviolet spectra were also found in coconut meat. Wheat leaves floated on dilute solutions (optical density at 260 mμ approx. 0.1) of these substances remained green for about two weeks while leaves floated on water turned yellow.

After precipitation of the purines the supernatants from wheat leaves and coconut meat were treated with charcoal deactivated with stearic acid. The charcoal was eluted with 8% phenol. After removal of phenol the eluate was chromatographed on paper in isopropanol: ammonia: water (10:1:1). The water eluate from the zone corresponding to an Rf value of 0.4-0.5 was active in the Avena straight growth test but was negative to both Ehrlich and Salkowski reagents. Alkaline hydrolysis of the eluate gave rise to indoleacetic acid and other Ehrlich positive compounds. A substance resembling arabinose in its Rf value and reaction with aniline phthalate was also produced.

10:30. LEFEVRE, CECIL W. and C. O. CLAGETT, The Pennsylvania State Univ. Further studies on the growth inhibitor from quackgrass.

Quackgrass *(Agropyron repens)* extracts inhibit the growth of agronomic species when applied to germinating seed. By means of paper chromatography, ion exchange, and silica gel chromatographic procedures several fractions have been concentrated which possess this inhibitory power. Preliminary chemical studies indicate an acidic component which is relatively unstable in acidic and basic solutions. Ultraviolet absorption data suggest that it is not aromatic. Infra-red spectra indicate it is of relatively low molecular weight, contains a carbonyl group and is probably carbohydrate in nature.

10:45. MICHEL, B. E., Univ. of Georgia, Athens, Ga. A plant growth inhibitor from cabbage.

Inhibitory action was first noted in the neutral fraction of ether extracts of etiolated cabbage seedlings, which inhibited the elongation of cabbage hypocotyl sections but stimulated that of oat coleoptile sections. Appreciable inhibitory activity could not be detected on paper chromatograms. This and other experiments indicated a volatile inhibitor. Steam distillates of cabbage seedlings contained an inhibitor of both cabbage and oat sections. It is probable that indoleacetonitrile was responsible for the earlier stimulation of coleoptile sections as they are quite sensitive to it, while cabbage sections show practically no response below 10^{-5} M.

Recently, by repeated steam distillation, about 0.1 ml of a light yellow oil with a density slightly greater than 1 was isolated from 3 kg of cabbage seedlings. Gas chromatography indicated 90 per cent or more of a single component. At present writing, based on odor, content of nitrogen, and existing literature, beta-phenethyl isothiocyanate is suspected. Work is continuing toward positive identification and determination of what quantities, if any, of the free isothiocyanate (usually believed to be present as the glucoside) may be present in tissues of various parts and ages of cabbage seedlings.

SESSION A4. Room 170, Electrical Engineering

Symposium: WEATHER AND ORGANISMS

Sponsored jointly by The Botanical Society of America, The American Society of Plant Physiologists and The Ecological Society of America

F. W. Went, *presiding*

9:00. WAGGONER, P. E., Connecticut Agricultural Experiment Station. Introduction. "What is agricultural meteorology?"

9:45. BENTON, G. S., Johns Hopkins Univ. The physics of water and its meteorological implications.

10:30. Recess.

10:45. RUSSELL, M. B., Univ. of Illinois. Water in the soil. Hydrology and availability of water to the plant.

11:30. Discussion.

MONDAY AFTERNOON
CONCURRENT SESSIONS B1, B2, B3, B4

SESSION B1. Room 310, Memorial Center

PHOTOSYNTHESIS II

M. Gibbs, *presiding*

1:30. FEWSON, CHARLES A., PAUL K. KINDEL and MARTIN GIBBS, Cornell Univ., Ithaca, N.Y. The role of fructose 1,6-diphosphate aldolase in photosynthesis.

Extracts of *Anacystis* (Richter, 1960) and extracts of red algae (Jacobi, 1959) lack aldolase. Using 4 different assays, we have confirmed this finding of Richter and extended it to *Anabaena* and *Nostoc*. A red alga, *Chondrus*, contained high amounts of aldolase. All conditions tried (alumina grinding, acetone powder, Mickle disintegration, fractionation at various pH with ammonium sulfate, treatment of particles with Tween 80 and digitonin, inhibition studies with other aldolases, addition of divalent cations) yielded negative results with the blue-greens. While *Chlorella* and *Scenedesmus* had high amounts of F-6-P kinase, triose phosphate dehydrogenases, and alkaline (pH 9) and acid (pH 6.0) FDP phosphates in comparison to G-6-P dehydrogenase and 6-PG dehydrogenase, the reverse was found with *Anacystis, Anabaena* and *Nostoc*. The blue-greens had a high amount of acid FDP phosphatase related to the basic phosphatase while the reverse was observed in the green algae. An asymmetric distribution of tracer was observed in glucose isolated from *Anacystis* after 40 second and 60 second photosynthesis in $C^{14}O_2$. These findings suggest a reevaluation of the role of aldolase in photosynthesis and a reevaluation of the PGA $\longrightarrow$ hexose route in photosynthesis.

1:45. HAVIR, EVELYN A. and MARTIN GIBBS, Cornell Univ., Ithaca, N.Y. Distribution of tracer in some products of $C^{14}O_2$ fixation by a reconstituted chloroplast system.

Protein extracted from spinach chloroplasts and combined in the presence of cofactors can bring about incorporation of $C^{14}O_2$ into a number of compounds including sugar phosphates. The products of $C^{14}O_2$ fixation obtained with this reconstituted system have been degraded to determine if the same labeling pattern is obtained as with the intact preparations.

Protein was extracted from lyophilized pea chloroplasts (isolated in organic solvents) and grana were prepared from pea chloroplasts isolated in 0.35M NaCl. Three experiments were run for forty minutes. The components, in addition to photosynthesis triphosphopyridine nucleotide reductase, TPN, ADP, and inorganic phosphate, were: (1) ribose-5-phosphate plus $HC^{14}O_3^-$, (2) ribulose diphosphate plus $HC^{14}O_3^-$, (3) carboxyl labeled phosphoglyceric acid. The radioactive compounds were separated by gradient elution on a Dowex Cl^- column. Glucose-6-phosphate, phosphoglyceric acid and fructose diphosphate were degraded.

Glucose-6-phosphate was labeled in the following way: C-1 5%, C-2 2%, C3 35%, C-4 58%, C-5 plus C-6 less than 1%. The labeling pattern was approximately similar in the three experiments. Phosphoglyceric acid from experiments (1) and (2) had 97% of the activity in the carboxyl carbon, 1% in the α-carbon and 2% in the β-carbon. The phosphoglyceric acid from experiment (3) had 91% in the carboxyl carbon, 4% in the α-carbon, and 5% in the β-carbon. The fructose diphosphate samples in the three experiments showed almost equal labeling in C-3 and C-4 while C-1 and C-2 were more active than C-5 and C-6.

In these experiments, there is very little migration of tracer from C-3 and C-4 of the hexose into C-1, C-2, C-5, C-6 as compared to that in the intact chloroplast. Mechanisms which could account for the difference in labeling between glucose-6-phosphate and fructose diphosphate will be discussed.

2:00. DAS, M. L. and F. L. CRANE, Purdue Univ., Lafayette, Ind. Low triphosphopyridine nucleotide levels in spinach chloroplasts.

Studies designed to examine the binding of TPN+ or TPNH in chloroplast with an improved method, developed in this laboratory, gave unexpectedly low amounts of TPN+ in chloroplast. The amount of TPN+ found in whole chloroplast and broken chloroplast were approximately 1×10^{-4} and 3×10^{-5} μmole respectively per μmole of chlorophyll. The TPN+ present can be washed out by exposure to hypotonic media.

It has also been found that the partially purified photosynthetic pyridinenucleotide reductase described by San Pietro and Lang (*J. Biol. Chem.* 231, 211, 1958) is capable of catalysing the light dependent reduction of DPN+ by chloroplast fragments even though only traces of TPN+ remain in the broken chloroplast. Addition of TPN+ to the reconstituted system consisting of broken chloroplast and dialysed reductase does not increase the rate of DPN+ reduction in light. Furthermore, the relative rate of DPN+ reduction is higher with respect to the rate of TPN+ reduction when a crude extract of chloroplast is used instead of the partially purified enzyme.

These results indicate that there is a separate pathway for reduction of DPN+ by chloroplasts in which TPN+ is not an intermediate reductant. This alternate pathway is probably catalysed by a DPN+ specific photosynthetic pyridine nucleotide reductase.

2:15. BLACK, CLANTON C., JOHN F. TURNER and MARTIN GIBBS, Cornell Univ., Ithaca, N.Y. Effect of light intensity on photosynthetic processes in spinach chloroplasts.

It is generally accepted that CO_2 assimilation by photosynthesis in plants is dependent on the formation of adenosine triphosphate (ATP) and reduced triphosphopyridine nucleotide (TPNH). This investigation describes a study of the effect of light intensity on CO_2 fixation, TPN reduction and ATP formation in spinach chloroplast preparations. At low light intensities there was a lag in CO_2 fixation but with further increase in light intensity there was rapid CO_2 assimilation. TPN reduction increased in an approximately linear fashion with increase in light intensity until saturation values were approached. ATP formation at low light intensities shows a similar lag to that observed with CO_2 fixation. At high light intensities the ratio of ATP to TPNH formed approximated to one but at lower light intensities the value fell to a low level. It is suggested that at low light intensities ATP formation may be a limiting factor in CO_2 fixation by chloroplasts.

2:30. KAHN, J. S. and A. T. JAGENDORF, Johns Hopkins Univ., Baltimore, Md. The ATP-ADP exchange enzyme from chloroplasts: Antigenic properties.

In an effort to determine the role of the ATP-ADP exchange enzyme (*J. Biol. Chem.* 236, 940 [1961]) in photophosphorylation, an attempt was made to produce an antibody to the purified enzyme by injecting it into rabbits.

The antibody obtained turned out to have rather unusual properties, in that the complex enzyme-antienzyme was both soluble and enzymatically active. Moreover, when this complex was precipitated, the precipitate retained the enzymatic activity.

Efforts are continuing to determine the role of the enzyme in photophosphorylation. Preliminary experiments suggest that it may function in the transfer of high energy phosphate from the ATP precursor to ADP (*Biochem. Biophys. Res. Comm.* 2, 259 [1960]).

2:45. KRALL, A. R., Univ. of Miami, School of Medicine, Miami 36, Fla. Properties of a phosphoprotein from spinach chloroplasts.

A chloroform extract of chloroplasts contains a protein that binds inorganic phosphate spontaneously. After heat denaturation the phosphate bond is stable to acid and labile to alkali. After partial acid hydrolysis, a phosphate containing peptide may be isolated by chromatography on Dowex-50. Phosphate binding by the extract under different concentrations of ADP, ATP and Mg^{++} can be best explained by the following formulation of the reaction:

(1) $Pi + Protein \rightleftarrows P \sim Protein$

(2) $P \sim Protein + ADP\ Mg^{++} \rightleftarrows Protein + ATP$

The "Protein" on the left side of equation (1) must necessarily be at a higher energy level than that on the right of (2). No direct evidence is yet available on that point. It is probable that the second reaction is catalyzed by a kinase system. As yet we have very little evidence as to whether 1 or 2 or more proteins are involved in the system. (This investigation was supported in part by a PHS research grant RG-8510.)

3:00. Recess.

3:15. JAGENDORF, A. T., The Johns Hopkins Univ., Baltimore, Md. Uncoupling chloroplasts by removal of cations.

During attempts to prepare phosphorylating fragments of spinach chloroplasts we observed major losses of activity following suspension in water. The cause of damage was found to lie in the removal of cations from chloroplasts previously exposed to EDTA, rather than to a simple physical disruption. Inactivation was prevented by all divalent cations at .0001 M or monovalent cations at .01 M. Accompanying the loss of phosphorylating ability was a marked activation of the ferricyanide—Hill reaction, especially of a rate-limiting "dark" step. By varying the composition of the medium it is possible to obtain varying degrees of uncoupling with a high degree of reproducibility.

3:30. GOOD, N. E., Pesticide Research Institute, London, Ontario. Uncoupling of the Hill reaction from photophosphorylation by anions.

The rate of reduction of oxidants by illuminated chloroplasts is stimulated 10- to 15-fold by the presence of high concentrations of many anions. This stimulation is associated with, and quantitatively related to, a decrease in the ability of the chloroplasts to phosphorylate ADP. Phosphate, pyrophosphate, arsenate, citrate, lactate and malonate are particularly effective uncouplers. Acetate and butyrate uncouple but also produce rapidly increasing inhibitions. Most of the polyvalent anions studied produce two rate-maxima but not two uncoupling-maxima when concentrations are varied; it seems that the intermediate concentrations responsible for relatively low Hill reaction rates are for some obscure reason inhibitory. The polyvalent anions are also the most powerful uncouplers, phosphate and citrate often producing measurable uncoupling effects when less than millimolar. Sulfate and chloride are themselves feeble uncouplers but interact in a complex fashion with other anions, sometimes causing thereby major rate changes. Zwitterions in the form of neutral amino acids apparently do not uncouple. Consequently we have prepared a neutral amino acid with appropriate dissociation characteristics to serve as a buffer for chloroplast preparations. Reacting tris(hydroxymethyl)aminomethane with chloroacetic acid gave tris(hydroxymethyl)methylglycine, pK's about 7.9 and 2.3. Preliminary tests indicate that this buffer is much less inhibitory at high concentrations than the parent tris(hydroxymethyl)aminomethane. Moreover, unlike the latter substance, it is used without associated anions.

3:45. STILLER, MARY, Univ. of Chicago, Chicago 37, Ill. Phosphorylation accompanying ferricyanide photoreduction by spinach chloroplasts.

A study has been made of the conditions under which phosphorylation can be elicited during the Hill reaction with ferricyanide. Both ferricyanide reduction and the accompanying phosphorylation have pH-activity curves with optima around pH 8 at 15°C; both activities are unstable at alkaline pH, so that the optima are greatly influenced by the time and temperature of the assay. Aged chloroplasts are more sensitive to alkaline conditions than are fresh preparations.

The addition of Mg^{+2}, ADP and orthophosphate to the Hill reaction system can elicit excellent rates of phosphorylation without appreciable effect on the Hill reaction rate. This null effect is the resultant of an inhibition of ferricyanide photoreduction by ADP and the relief of this inhibition by Mg^{+2} and phosphate; at the same time, the addition of these reagents induces phosphorylation. Arsenate and sulfate are also effective in relieving the inhibition by ADP. High concentrations of phosphate and arsenate (but not sulfate) added to the ADP-inhibited system stimulate it to rates higher than the control rate of ferricyanide reduction. Both the inhibition and the stimulation are small (about 30%) at 15°; at 23° these effects may appear to be much larger, but the chloroplasts are extremely unstable. At 15° or 23°, with fresh or aged preparations, the increment of ferricyanide photoreduction obtained upon addition of Mg^{+2} and phosphate to the ADP-inhibited system, is equal, on a molar basis, to the phosphorylation thereby induced.

4:00. KROGMANN, D. W. and M. L. STILLER, Wayne State Univ., Detroit 2, Mich. and The Univ. of Chicago, Chicago, Ill. Naturally occurring cofactors for photophosphorylation.

When spinach chloroplasts are isolated in an aqueous medium by conventional procedures, a catalytic amount of a redox cofactor, such as FMN, or a stoichiometric amount of suitable Hill oxidant, such as TPN, must be added to the chloroplasts to elicit photophosphorylation. The naturally occurring components which act in these capacities have presumably been washed out of the chloroplasts by the isolation procedure. Chloroplasts isolated in non-aqueous solvents from lypholyzed leaves have been found to yield a water soluble, heat stable extract which will support photophosphorylation when added to chloroplasts prepared in aqueous media. The phosphorylation elicited by this extract of non-aqueous chloroplasts is oxygen dependent and proceeds at a rate as high as 250 micromoles ATP synthesized per mg. chlorophyll per hour. Chromatography of the cofactor containing extract indicates that there are several active fractions.

A number of flavone glycosides and, phenolic compounds, which occur naturally in leaves, support photophosphorylation in a manner quite similar to that exhibited by the extract of non-aqueous chloroplasts.

4:15. WHATLEY, F. R., A. A. HORTON and DANIEL I. ARNON, Univ. of California, Berkeley 4, Calif. Noncyclic photophosphorylation by chloroplasts, with and without oxygen evolution.

The hypothesis was advanced by Hill, Burk and Warburg that the oxygen evolved in photosynthesis represents the difference between the total oxygen formed photochemically and the oxygen consumed in a simultaneous dark reaction; they proposed that the dark reaction is necessary to supply additional energy for the assimilation of CO_2. When later, other investi-

gators found that ATP formation in noncyclic photophosphorylation increases oxygen evolution in the Hill reaction—a result opposite to that expected from the above hypothesis—Hill accepted the new evidence and rejected his earlier hypothesis, whereas Warburg retained the hypothesis and attributed the increased oxygen evolution observed by other investigators to uncontrolled pH changes. We have now reinvestigated the effect of photophosphorylation on oxygen evolution by chloroplasts at different hydrogen ion concentrations in well-buffered reaction mixtures. Our results, in agreement with the observations of Avron, Krogmann and Jagendorf, and of Davenport, show that an increase in oxygen evolution occurs over a range of pH when ATP formation is coupled with a photoreduction of TPN or ferricyanide.

We regard oxygen as an excreted by-product liberated during green plant photosynthesis when water (OH^-) is used as the terminal electron donor. When we experimentally substituted ascorbate for water (OH^-) as the electron donor, we found that isolated chloroplasts carried out a type of noncyclic photophosphorylation, similar to that observed in this laboratory with chromatophores of *R. rubrum:* photoreduction of pyridine nucleotide was coupled with ATP formation, but no oxygen was liberated.

4:30. HOCH, G., M. J. RUSSO and BESSEL KOK, RIAS, Baltimore, Md. Sensitization of chloroplast phosphorylation.

Studies have been made of the relative efficiency of various wavelengths of red light in sensitizing chloroplast phosphorylation. Dense chloroplast suspensions were employed to "totally absorb" the incident light. Relatively high intensities of monochromatic light were obtained by interference filters. At high light intensity maximal phosphorylation efficiency occurred at approximately 710 mμ when phenazine methosulphate or reduced 2,6-dichlorophenol indophenol are employed as cofactors. This maximum was not present when menadoine or flavin mononucleotide were used as the catalytic cofactor. With low intensities of light the rate in the far red ($\lambda > 690$ mμ) fell more rapidly for flavin mononucleotide phosphorylation than for phenazine methosulphate.

Addition of shorter wavelengths to far red light caused a phosphorylation rate greater than the sum of the individual beams when flavin mononucleotide was used as the cofactor. No "enhancement" was found with phenazine methosulphate. These results will be discussed in terms of a two quanta hypothesis for photosynthesis.

SESSION B2. Room 4, Memorial Center

GROWTH REGULATORS II

A. C. Leopold, *presiding*

1:30. KEY, J. L., Univ. of California, Davis, Calif. The relationship of ascorbic acid to the growth regulating action of auxin.

In a previous study ascorbic acid was identified as a compound which accumulated in etiolated soybean seedlings which had been treated with 2,4-D. Subsequent work revealed changes not only in the reduction level of ascorbic acid but also of soluble and protein sulfur. The present report deals with results from a detailed study of the effects of a wide range of growth regulators on the ascorbic acid content of cucumber plants. Spray treatment of these plants with IAA or synthetic auxins (2,4-D, etc.) leads to inhibition of further leaf growth and apical meristem activity associated with a promotion of expansion and cell division in the stem tissue. The magnitude and duration of these responses depends upon the chemical used and concentration of the chemical. In response to auxin the ascorbic acid concentration decreases markedly in the inhibited leaf tissue while showing large increases in the stem tissue where growth is promoted. There is a good correlation between activity of the chemical used and the ascorbic acid response. These results will be discussed in relation to the action of auxin.

1:45. DYCUS, AUGUSTUS M., Arizona State Univ., Tempe, Ariz. The action and interaction of indoleacetic acid and gibberellic acid on zinc deficient bean plants.

The growth of *Phaseolus vulgaris,* L (variety black valentine), in a nutrient solution lacking the metal zinc shows the characteristic symptoms of shortened internodes and small mottle-leaf of zinc deficiency. Young plants respond quickly to addition of 0.001 ppm zinc in the nutrient solution or 0.05 ppm I.A.A. in the nutrient solution.

Seedlings transplanted to nutrient solutions minus zinc but with gibberellic acid added show the stem growth response normally associated with gibberellin but the leaves remain small and mottled. The fresh and dry weights of the gibberellin treated zinc deficient plants were less than the zinc deficient plants. The zinc deficient plants showed a smaller fresh and dry weight when compared to the plants grown in full nutrient solution.

Cell counts and measurements were made of longitudinal and cross sections of normal, zinc deficient and zinc deficient gibberellin treated plant stems. The evidence indicates that I.A.A. and gibberellin stimulate two distinctly different mechanisms of bean stem elongation.

2:00. REINHARD, E. and A. LANG, California Inst. of Technology, Pasadena, Calif. Natural gibberellins in *Hyoscyamus niger* in relation to development especially to flower formation.

The content of *Hyoscyamus niger* plants (a long-day variety and a cold-requiring variety) in native gibberellins was determined at several stages of development. The LD-variety was extracted in the vegetative state and after exposure to different numbers of long days (LDs). The activity was mainly determined by means of a dwarf-pea test; comparative activity tests were done on maize dwarf mutants and cucumber seedlings. Only one gibberellin fraction (eluable with 60 per cent acetone, using a charcoal-celite column as means of chromatography) was found in vegetative plants and after 5 and 10 LDs. None was present after 30 LDs. After 20 LDs the amount of gibberellin was markedly increased and 4 different gibberellin fractions (eluable with 50, 60, 70, 90 per cent acetone) could be separated.

The cold-requiring variety was extracted before cold treatment (CT), immediately after CT and different numbers of days later. After CT part of the plants were kept on short day (SD) and part on LD. Before CT one gibberellin (60 per cent

acetone) was present. None could be detected immediately and 2 and 4 days after CT. Eight days after CT high amounts of 4 different gibberellins—apparently the same as in the LD strain after 20 LDs—were found in LD-exposed plants; a low amount of the "60 per cent" gibberellin was present in SD-treated plants. After 20 LDs only one gibberellin ("60 per cent") was present; the level was similar to that in non-cold-treated plants.

Thus in both varieties similar quantitative and qualitative changes of the gibberellin composition do occur coincident with the start of bolting and the early stages of stem elongation. The relations between gibberellin content and development will be briefly discussed.

2:15. DUYSEN, M. E., Univ. of Nebraska, Lincoln 8, Nebr. A quantitative comparison of methods of applying gibberellic acid to plants.

Plants of sage, *Salvia officinalis,* and poinsettia, *Euphorbia pulcherrima* 'St. Louis,' were grown in the greenhouse in washed river sand and in potting soil comprised of one part river sand blended with one part of prairie loam. Gibberellic acid as "Gibrel" was applied (1) as a spray (100 or 1000 μg/plant), (2) in a lanolin ring (100 or 10,000 μg/plant), (3) as a drop of solution on the tip of the shoot (100 μg/plant), and (4) with the nutrient solution (100 or 1000 μg/plant). In addition the hollow stem of poinsettia was injected with solution (100 μg/plant).

The relative effectiveness of the several methods of application on stem height, stem diameter, internodal length, percentage of dry matter, and shoot-root ratios was statistically analyzed by the Duncan multiple range test. Gibberellic acid was most efficiently used when applied either as a spray or to the stem tip of sage and when injected in poinsettia. A more effective response was found for plants grown in sand than in soil with differences in dosage levels producing variable results.

2:30. KATO, J., W. K. PURVES and B. O. PHINNEY, Univ. of Osaka Prefecture, Sakai, Japan, and Univ. of Calif., Los Angeles, Calif. Gibberellin-like substances from cryptogams.

Gibberellins and gibberellin-like substances have been found in many families of the Angiospermae, but little is known about their occurrence in Cryptogamae other than the Ascomycete *Gibberella fujikuroi.* We have surveyed the occurrence of gibberellin-like substances in various cryptogams including Gymnospermae, Filicinae, Bryophyta, Chlorophyta, Phaeophyta, and Schizophyta. Active extracts are described in terms of their specificity towards three single gene dwarf mutants of *Zea mays.* Included are the first reports of gibberellin-like substances from Gymnospermae and Filicinae. An extract of *Juniperus chinensis torulosa* fruit was inactive on the mutant *dwarf-1* but active on *dwarf-3* and *dwarf-5,* while immature fronds of the tree fern *Alsophila australis* yielded an extract active on all three mutants.

2:45. MARUMO, SHINGO and ROY W. CURTIS, Purdue Univ., Lafayette, Ind. Chemical studies on malformin.

Two kinds of malformin, which induce curvatures and grotesque malformation on bean plants, were isolated from the culture filterates of *Asp. niger* and the names, old and new malformin, have been proposed tenatively for these compounds.

Old malformin, $C_{23}H_{39}N_5O_5S_2$, is a neutral cyclic pentapeptide consisting of D-leucine (1), L-isoleucine (1), L-valine (1) and half D-cystine (2). Employing the 2,4-dinitrophenylation method, the data obtained from the partial hydrolyzates of old malformin, its oxidative product and its desthio-product suggested the following amino acid sequence: D-leucyl-L-isoleucyl-D-cysteinyl-L-valyl-D-cysteine anhydride.

3:00. Recess.

3:15. ZAROOGIAN, GERALD E. and ROY W. CURTIS, Purdue Univ., Lafayette, Ind. Comparison of plant regulators produced by *Arachniotus trisporus* and *Penicillium thomii.*

Production of plant regulators by *Penicillium thomii* and *Arachniotus trisporus* occurred during autolysis of the mycelium. A bioassay, employing Black Valentine bean seedlings, was devised. Under certain conditions, the length of the unaffected lateral leaflet/length of the inhibited lateral leaflet (ratio of the length of the lateral leaflets) was proportional to the amount of plant regulator applied to one of the cotyledons. Most of the evidence obtained by ion-exchange and paper chromatography indicated that the plant regulators produced by *A. trisporus* and *P. thomii* were identical or closely related. It appears that the plant regulators are responsible for a purple coloration when ferric chloride is added to culture filtrates of these fungi.

3:30. BUSHNELL, W. R., USDA, Univ. of Minnesota, St. Paul 1, Minn. Green islands and associated symptoms in wheat leaves produced by kinetin, benzimidazole, nickel or cobalt.

The possibility that the green islands produced in senescent leaf tissue around rust and mildew infections are the result of the action of a chemical agent was investigated indirectly by testing a wide variety of materials for the property of inducing similar localized retention of green pigments in detached wheat leaves. Benzimidazole, nickel sulfate, nickel chloride, or cobalt chloride applied as 5 ul drops at a concentration of 10^{-3}M and kinetin at 10^{-4}M produced green islands about 1 cm long. The islands became visible 3 to 4 days after these solutions were applied. Yeast extract, proteose peptone, and beef extract also produced green islands. The ash of beef extract induced green islands while the ash of yeast extract and proteose peptone did not.

All substances that produced green islands induced local deposits of starch, maximum starch development occurring 2 to 3 days after treatment. Further studies with nickel, benzimidazole, and kinetin indicated that green island formation was preceded by localized increases of 10 to 30% in dry weight and nitrogen content.

Nickel, benzimidazole, and kinetin were less effective in inducing changes in attached leaves than in detached leaves. At high concentrations these compounds did induce localized starch deposition in attached leaves, but only in trace amounts and in irregular patterns. Green islands, however, developed in attached leaves 2 to 3 weeks after application of kinetin.

Whether materials of the types represented here are active in rusted or mildewed tissue is now under investigation.

3:45. KIM, WON KYUM and V. A. GREULACH, Univ. of North Carolina, Chapel Hill, N.C. Promotion of algal growth by IAA, GA and kinetin.

IAA promoted the growth of *Anabaena variablis* at 1 to 25 ppm with maximum effect to 10 ppm and inhibited growth at 50 and 100 ppm as measured by dry weight increase. The growth of *Chlorella pyrenoidosa* was promoted by 10 ppm IAA on the basis of optical density, dry weight and cell number, but at 5 ppm only cell number was increased. At 1 ppm there was no effect while 25, 50 and 100 ppm had increasing growth-inhibiting effects. *Oedogonium cardiacum* filament length was increased by 5, 10 and 20 ppm IAA, 10 ppm being as effective as 20 ppm. There was no significant influence of 1 or 50 ppm. Only 10 ppm IAA was used on *Vaucheria sessilis* and it increased filament length significantly. IAA and GA had an additive effect on the growth of both *Chlorella* and *Oedogonium*, promoting both cell division and cell elongation in the latter. IAA partially overcame the growth inhibition of both species produced by MH or 2,4-D, again influencing both cell division and cell elongation in *Oedogonium*.

GA promoted *Anabaena* growth more at 25 ppm than at 50 ppm, while 1 and 10 ppm had no effect. In *Chlorella* 5, 10 and 20 ppm promoted growth in increasing order but 50 ppm had no effect, while 1 ppm increased cell number but not optical density or dry weight. *Oedogonium* filament length was increased by 5, 10 and 20 ppm in order, while 1 and 50 ppm had no effect. GA at 10 ppm increased *Vaucheria* filament length. GA and kinetin had an additive effect on *Chlorella growth*, while GA partially overcame the growth inhibition induced by MH, 2,4-D and 5-FU. In *Oedogonium* GA partially counteracted 5-FU growth inhibition and MH inhibition of cell division but not MH inhibition of cell elongation.

Kinetin promoted *Anabaena* growth at 25 and 50 ppm, inhibited at 100 ppm and had no effect at 1 and 10 ppm. In *Chlorella* 1, 5, 10 and 20 ppm promoted growth in increasing order while 50 and 100 ppm inhibited. In *Oedogonium* 1 ppm had no effect but 5, 10, 25 and 50 ppm promoted growth with the maximum at 25 ppm. Kinetin partially overcame the growth inhibition of *Chlorella* brought about by MH, 5-FU or 2,4-D.

All differences mentioned were highly significant statistically as determined by calculation of t values and standard deviation.

4:00. WITTWER, S. H., Michigan State Univ., East Lansing, Mich. Some effects of kinetin on the growth of intact plants.

There are few reports concerning the effects of kinetin and related compounds on the behavior of intact plants. Foliar applications or soil treatments, usually limited in dosage by low water solubility of the compound, have been generally ineffective. Kinetin added to the root medium of plants growing in solution cultures, however, has pronounced effects upon a wide variety of crop plants. Gradations in growth and other responses usually occur at concentrations between 10^{-5} and 10^{-8} molar. Root-top ratios are increased, and pseudonodules occur on greatly enlarged lateral roots. Flowering is accelerated in Alaska peas under short days, but greatly delayed in the tomato. The effects of kinetin are opposite to those of gibberellin in flowering and internode elongation of dwarf peas, but in overcoming some of the growth suppressing effect of 2-chloroethyltrimethylammonium chloride, gibberellin and kinetin are similar.

4:15. RUBINSTEIN, B. and A. C. LEOPOLD, Purdue Univ., Lafayette, Ind. Internal factors controlling bean leaf abscission.

An examination of the effects of various treatments and substances on leaf abscission in red kidney bean has been carried out using the petiole explant test. Four types of stimulations of abscission are found. Auxin stimulates abscission when its concentration is low as reported earlier. A signal for abscission is provided by the stem apex, as indicated by the fact that decapitation of the stem prevents leaf abscission, and the time of this decapitation is reflected in the inherent tendency of explants from leaves of the decapitated plants to abscise. Extracts from senescent leaves are found to be effective in stimulating abscission as expected from the earlier reports of Osborne and Biggs. Amino acids are also very effective especially ethionine and alanine at concentrations of 5×10^{-4} and 5×10^{-3}M. These four types of abscission stimulations are compared, and it is suggested that amino acids exported from the leaves participate in triggering the abscission process.

SESSION B3. Room 318, Memorial Center

MINERAL NUTRITION

J. Skok, *presiding*

1:30. TIFFIN, LEE O., and JOHN C. BROWN, U. S. Dept. of Agriculture, Beltsville, Md. Iron chelates in plant exudate.

Soybean stem exudates contain compounds which bind Fe whether the Fe is absorbed through the roots or added to exudate *in vitro*. These substances maintain Fe in soluble form at pH 8.6, move with Fe to the anode, are stable to heat, and are not sedimented at 100,000 x g.

By use of organic buffers, Fe^{59} in the exudate was separated electrophoretically into bands which were located by X-ray film. The buffer was removed from the paper and the iron eluted with water. The Fe in the water extract separated as before when subjected again to electrophoresis.

By subjecting the water extracts to paper chromatography it was shown that Fe^{59} remained at the origin. However, two exudate acids moved from the origin to positions alongside known malic and malonic acids. The malic acid in the exudate was found at higher concentrations than the malonic.

Fe^{59} combined with known malic and malonic acids and run electrophoretically alongside the exudate unknowns further confirmed the presence of these two Fe chelates in the soybean exudate. These results indicate that malic and malonic acids may function in the transport of Fe within the plants.

A preliminary survey of plant species has shown that stem exudates of sunflower, cotton, and tomato contain Fe com-

pounds which move as anions and have mobilities very similar to the Fe compounds in the soybean exudate.

1:45. WALLIHAN, E. F., Univ. of California, Citrus Experiment Station, Riverside, Calif. Iron absorption as a function of root growth.

Such diverse conditions as high soil moisture, low soil oxygen, and presence of $CaCO_3$ are known to depress absorption of iron. It is found that they also reduce the root:top ratios of the plants.

This may mean that iron absorption is a function of root surface area which suggests, in turn, that intimate contacts between soil particles and the root surfaces are important to iron absorption. Evidence favoring this point of view will be discussed.

2:00. WALLACE, A., D. VAN NOORT, and C. B. JOVEN, Univ. of California, Los Angeles, Calif. Iron and manganese interaction on *in vitro* CO_2 fixation in leaf preparations with ribose-5-phosphate as substrate.

Although iron ($FeSO_4$) can inhibit the enzymatic reaction sequence that results in synthesis of 3-phosphoglyceric acid from ribose-5-phosphate and CO_2 (*Plant Physiol* 34:446-449), small amounts of iron stimulated the reaction in crude preparations of leaves of several plant species prepared with tris (hydroxymethyl) amino methane (tris) buffer. Removal of low-molecular weight materials by passing through a sephadex column did decrease the effect. The application of a similar amount of managnese ($MnSO_4$) (1 and 2 μM/ml reaction mixture) greatly inhibited the reaction but this could be reversed in part by the addition of $FeSO_4$. The effect of iron was less pronounced in the presence of EDTA, but EDTA itself greatly increased the reaction. Fe^{++} to a slight extent can replace Mg^{++} in the reaction but this was not the cause of the iron effect. Fe^{59} was partially bound to the protein as indicated by passing the preparations through a sephadex column but Mn^{54} was not except for a very slight extent. With phosphate buffer in place of tris buffer the iron, manganese, and EDTA effects were almost absent.

2:15. BELL, W. D. Pennsylvania State Univ., University Park, Pa. Influence of iron nutrition and the pH of the rooting medium on the phenotypic expression of the maize mutants.

Iron nutrition of two yellow-striped maize mutants, ys_1 and ys_3, has been compared. Foliar applications of iron salts in aqueous solutions evoke local tissue greening of soil-grown plants homozygous for either allele, that of genotype ys_1/ys_1 being more rapid than that of ys_3/ys_3. Seedlings of either genotype produce only green leaves when grown on a complete nutrient solution containing iron as Fe-HEDTA (feric chelate of N-hydroxyethylethylenediaminetriacetic acid). The lesion in iron metabolism of ys_1/ys_1 plants has been shown previously to involve absorption by the roots; that in ys_3/ys_3 plants seems to be more in translocation or utilization.

The pH of the nutrient medium influences the expression of several alleles on chromosome 2 in maize. In nutrient-solution cultures maintained at pH values of 5.5 to 7, more green than white leaf tissue was produced by plants homozygous for the albescent (*al*) allele and greening of virescent v_4/v_4 plants was retarded at the lower value. In contrast a new recessive phenotype, tentatively named target spot, was not displayed at pH 5.5 but appeared when the pH of the nutrient solution was raised to or maintained at 7.

This investigation will be extended to determine possible physiological interrelationships among these alleles and others as available.

2:30. WALKER, RICHARD B., Univ. of Washington, Seattle 5, Wash. Greenhouse studies of the mineral requirements of sitka spruce (*Picea sitchensis*).

Seedlings of sitka spruce, *Picea sitchensis*, were grown in solution and sand cultures using Hoagland-type solutions. Treatments involved "complete" solutions and low levels of Ca, Mg, K, N, P, S, Fe, Mn, and B. Rates of growth of the seedlings were followed for periods up to two years. Deficiency symptoms were observed in all of the low-level treatments, these symptoms will be described and illustrated. Chemical analyses of the foliage, stems, and roots made possible the estimation of deficiency levels and mineral requirements. These will be discussed and compared with the requirements of other coniferous species of the Pacific Northwest.

2:45. INSELBERG, EDGAR, Na-Churs Plant Food Co., Marion, O. Simultaneous determination of radioisotope pairs by the decay constant differentiation principle.

The simultaneous labeling of a biological system with two radioisotopes entails two principal advantages: the fate of two elements can be investigated under identical conditions, in the same experiment; and a saving of time and expense results from performing one experiment instead of two.

The counting rate of each of two radioisotopes in a mixed activity is resolved by assaying at two different times and solving a system of equations utilizing the difference in decay rates.

The precision indices of simultaneously determined nuclides can be expressed as functions of nine variables. The variability of each isotope, however, has a minimum only with respect to the time elapsed between the two counts of the mixed activity. Since the minima of the two isotopes do not coincide, optimum conditions can be attained by minimizing the sum of the coefficients of variation of the two isotopes and imposing the constraint that the ratio of the coefficients of variation will have some desired value.

The theoretical model was validated with a series of factorial experiments utilizing synthetic activities. The variability of a simultaneously determined isotope decreased as its relative abundance increased, with increasing length of counting and counting rate, and as the optimum time interval between counts of the mixed activity was approached. If the disparity in the half-lives of the tracer elements is considerable and near-optimum conditions are selected, simultaneous labeling can be used with relatively small decrease in precision.

3:00. Recess.

3:15. SKOK, JOHN, Argonne National Laboratory, Argonne, Ill. Effect of GA and light on the utilization of boron and the development of boron deficiency symptoms.

It has been postulated that boron is utilized in the formation of plant tissues and functions specifically in the maturation and differentiation of the cell. It should then be possible to modify the boron requirement by factors that affect growth. Sunflower plants grown with minimal amounts of boron were treated with gibberellic acid (GA) and others were grown under red or white light. Boron deficiency symptoms appeared earlier in plants treated with GA than in untreated plants and the appearance of symptoms was greatly delayed in plants grown under red light, as compared to those grown under white light. Since GA accelerates growth, particularly cell elongation, and red light depresses development and differentiation, these results further demonstrate the relationship of boron to cellular maturation and differentiation. (This work was performed under the auspices of the U. S. Atomic Energy Commission.)

3:30. STANLEY, ROBERT G., Pacific S.W. Forest and Range Exper. Sta., Berkeley, Calif. Effects of various forms of boron on germinating pollen.

Tributyl borate, sodium tetraborate, boric acid, potassium metaborate and tetraborate were found to stimulate germination of *Amaryllis hybrida* pollen at a concentration of 5 μg/ml. Tributyl borate gave maximum growth response and phenyl borate inhibited pollen growth at this concentration. Both boron and exogenous sugar were required to stimulate pollen growth. The ability of pollen to absorb different amounts of boron from these different sources of boron was followed using pine pollen. It was found that 100 mg of pollen absorbed 9.8 μg/ml of boron over 48 hours, but only 5.2 μg/ml boric acid solution. The relation of glucose-1-C^{14} oxidation was studied in the various boron media. Mechanisms for maintenance of germinative capacity in pollen in relation to cell carbohydrates, and the intracellular role of boron will be discussed.

3:45. KWACK, B. H. and J. L. BREWBAKER, Brookhaven National Laboratory, Upton, Long Island, N.Y. The essential role of calcium ion in pollen germination and the population effect.

The "population effect" or "mutual stimulation effect" occurs when pollen are cultured in standard media. As the number of pollen grains increases in a constant amount of medium, the germination percentage and tube lengths proportionately increase (18 species tested in this laboratory).

It was suspected that the population effect resulted from the equilibrations of a water-soluble pollen growth constituent ("PGF") in the grains and surrounding medium (10% sucrose and 100 ppm boric acid). Sub-optimal concentrations of PGF were believed to arise when a few grains were sown in culture. These suspicions were confirmed by showing that cell-free extracts from large pollen populations completely overcame the "population effect."

In a series of biochemical studies of pollen and plant extracts and of conventional growth constituents, calcium ion was found to be the PGF (15 species tested). Although calcium was not replaceable by ions such as strontium, other cations (Mg^{++}, K^+ or Na^+) were required to permit the calcium activity. These data will be discussed in relation to pollen growth phenomena, as of the incompatibilities in plants, and in relation to calcium ion and calcium-borate nutritional relationships.

4:00. MINSHALL, WM. H., Pesticides Research Institute, University Sub P.O., London, Ontario, Canada. The effect of nitrogen on root pressure and uptake of nutrient ions by tomato roots.

Coinciding with the diurnal rhythm of exudation from detopped stumps of tomato plants is a diurnal rise and fall in the concentration of amino acids and other constituents in the exudate. Urea applied to the soil of a potted tomato plant in the 10 to 12 leaf stage increases the midday rate of exudation to between 3 and 4 ml per plant per hour. During this stimulation the exudate contains increased quantities of amino acids, nitrate, phosphate, potassium, etc. The evidence will be examined for possible relationships between the classical root pressure mechanism, nitrogen metabolism in roots, and the uptake of such ions as potassium and phosphate.

4:15. VAADIA, YOASH, FRANKLIN C. RANEY and T. K. HODGES, Univ. of California, Davis, Calif. Dispersion of H^3 and transport of Cl^{36} in topped sunflower and grapevine plants.

Exudate of sunflower and grapevine was analysed for several days after H^3 and Cl^{36} were placed in the external medium. Curves illustrating the rate of appearance of H^3 and Cl^{36} are presented. Exudate concentrations of H^3 reach those of the external solution after ten hours or more depending on root size and exudation rate.

In low salt sunflower plants Cl^{36} concentrations in the exudate are several-fold higher than external concentration within thirty minutes. In high salt sunflower plants the rate of appearance of Cl^{36} is similar to that of H^3 and several hours elapse before external concentrations are reached. In grapevines, despite accumulation of Cl^{36} in the roots, no Cl^{36} could be detected in the exudate during the eight days of measurement.

4:30. O'KELLEY, J. C. and W. R. HERNDON, Univ. of Alabama, University, Ala. Alkaline earth elements on zoospore release and development in *Protosiphon botryoides*.

Cells of *Protosiphon botryoides* Klebs from depleted nutrient medium containing Ca were washed and resuspended in fresh complete medium with Ca; or in media with a Sr, Ba or Na replacement, respectively, for Ca; or in chloride solutions; or in deionized water; zoospore release was observed in these media upon illumination following a 12 hr dark period. Free zoospores were less abundant in Sr, Ba and Na replacement media than in the Ca medium. Zoospore production and release were also depressed in solutions of only $CaCl_2$ and in deionized water. In the Sr and Ba media, zoospores were formed but not released from the parent cell, as a rule; some zoospores were released in mass within a gelatinous vesicle which did not liquefy and set the zoospores free; these zoospores lost motility and continued development in Sr, producing characteristic spheroidal clusters of aplanospores. In the Na medium protoplasmic cleavage preceding zoospore formation was severely inhibited. A study of the reversibility of Sr inhibition of the zoospore release mechanism revealed evi-

dence of revision 12 hr after replacement of Sr by Ca. Walls of cells produced in Ca are rich in ruthenium red-positive materials, whereas cells produced under conditions of Sr replacement lack these materials. This study was supported by grant A-3680 Nutr. from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

SESSION B4. Room 170, Electrical Engineering

Joint Symposium: **WEATHER AND ORGANISMS (continued)**

F. W. Went, *presiding*

2:00. DAWSON, W. R., University of Michigan. The terrestrial animal in relation to water.

2:45. KRAMER, P. J., National Science Foundation. The plant in relation to water: physiological considerations.

3:30. Recess.

3:45. DECKER, J. P., U. S. Forest Service. The plant in relation to water: ecological considerations.

4:25. WENT, F. W. Summary of symposium.

MONDAY EVENING, AUGUST 28

6:30. Annual banquet of the American Society of Plant Physiologists. South Ballroom, Union.
A. W. NAYLOR presiding.

Stephen Hales award address: P. W. STOUT, Univ. of California.

9:00. General meeting for all participating societies. Hall of Music.

TUESDAY, AUGUST 29

TUESDAY MORNING
CONCURRENT SESSIONS C1, C2, C3, C4

SESSION C1. Room 4, Memorial Center

Informal Discussion: **CHEMOTROPISM AND CHEMOTAXIS**

W. G. Rosen, *presiding*

8:30.

SESSION C2. Room 310, Memorial Center

BIOCHEMISTRY II

J. F. Thompson, *presiding*

8:25. Announcements.

8:30. WANG, DALTON, Univ. of Manitoba, Winnipeg, Manitoba, Canada. Carbon metabolism of amino acids. I. Formation of sugars from carbon-14 labelled glycine in wheat leaves.

When leaves of Khapli wheat were fed with glycine-C^{14}, a considerable amount of labelled carbon was found in sugars. Both the methylene carbon and the carboxyl carbon of glycine were effectively used in the transformation of sugars from this amino acid. The rate of formation of sugars in light was many fold of that in dark. Dinitrophenol showed no significant effect on this transformation. A supply of non-radioactive glycolic or glyoxylic acids to the leaf increased the rate of transformation, yet the glyoxylic acid isolated as phenylhydrazone from this treatment did not show any higher radioactivity than the control. Evidence indicates that the carbon chain of glycine does not follow the path of glyoxylic-glycolic-acids in the formation of sugars.

8:45. ZACHARIUS, R. M. and S. KRULICK, Eastern Utilization Research and Development Div., USDA, Philadelphia 18, Pa. A quantitative investigation of the nitrogen fractions of the snap bean seed.

The protein and non-protein N (NPN) fractions of the snap bean seed *(Phaseolus vulgaris)* have been investigated by ion-exchange chromatography, paper chromatography and classical analytical methods. The NPN fraction contains 8.0-8.4% of the total N found in the whole bean. A third of this extractable N is Van Slyke N. The major components of the NPN are pipecolic acid, γ-L-glutamyl-S-methyl-L-cysteine, choline, asparagine, aspartic and glutamic acids. A considerable proportion of the NPN consists of non-protein amino and imino compounds. A quantitative analysis of the NPN and protein N fractions is presented. Evidence is advanced for the presence of some non-protein amino acids hitherto not reported in the bean seed.

9:00. MANS, R. J., Biology Div., Oak Ridge National Laboratory, Oak Ridge, Tenn. Factors involved in the cell-free incorporation of amino acid into maize protein.

Particle systems capable of incorporating radioactive leucine into protein have been isolated from fresh maize kernels and developing maize seedlings. The requirements and characteristics of the two systems are quite similar and differ but slightly from those of the classical system isolated from rat liver. A microsomal pellet isolated from homogenates by centrifugation at 100,000 X *g* for 90 minutes requires the addition of a high speed supernatant, adenosine triphosphate, an energy generator, magnesium, and guanosine triphosphate in

order to incorporate C^{14}-leucine. The degree of maturity of both the kernel and the seedling is critical to the isolation of active materials. In the kernel, the endosperm and the embryo are both active, but at different stages of kernel development. In the etiolated seedling, the plumule and scutellum yield the most active preparations; the more readily accessible plumule is used for routine preparations. Kinetic experiments conducted with deoxycholate treated particles demonstrate that the particle protein is rapidly saturated with C^{14} -leucine and that the radioactivity remains associated with the particles throughout the incubation period. The possible role of soluble ribonucleic acid as well as other cellular components in the incorporation of amino acids into maize protein is currently under investigation.

9:15. EILBERG, RALPH and C. O. CLAGETT, The Pennsylvania State Univ., University Park, Pa. Incorporation of naturally occurring peptides into wheat ribosomes.

Wheat plants at the three leaf stage were exposed to $C^{14}O_2$ in the light for 30 minutes. The naturally occurring labeled peptides were isolated by extraction with alcohol, chromatographed and recovered from the paper. Using ribosomes isolated from wheat plants of the same age, and the necessary cofactors in an *in vitro* cell free system, it was possible to show incorporation of these labeled peptides into the ribosomal nucleoprotein. Further experiments showed that the peptides were incorporated much faster than amino acids. Peptide incorporation was inhibited partially by the addition of small amounts of DL-amino acids to the peptide incorporating system. The observed inhibition appears to be due to the D-amino acid moity. In several experiments in which the L-amino acids were added to the peptide incorporation system, little or no inhibition was observed.

9:30. MORRIS, CLAYTON J. and JOHN F. THOMPSON, USDA, Ithaca, N. Y. Some new γ-glutamyl dipeptides from plants.

The first γ-glutamyl dipeptide isolated at this Laboratory was obtained from the alcohol extract of red kidney bean seeds. It was later crystallized and proven to be γ-L-glutamyl-S-methyl-L-cysteine by elemental analysis, degradation and synthesis.

Two new peptides were found in the bulbs of Wedgewood iris. One was the γ-glutamyl dipeptide of $(-)$ β-amino-isobutyric acid and the identity of the second was established by elemental analysis, degradation and synthesis to be γ-glutamyl-β-alanine.

The identity of two more peptides, found in soybeans, has been definitely established as γ-L-glutamyl dipeptides of L-tyrosine and L-phenylalanine respectively while two more have been tentatively established through chromatographic evidence as glutamyl peptides of methionine and leucine.

9:45. OAKS, A. and H. BEEVERS, Purdue Univ., Lafayette, Ind. The nitrogen balance in excised maize embryos.

Excised maize embryos (plus scutellum) unlike those from barley and oats grow well in a glucose-salts solution. After 4 days in culture the dry weight is similar to that of the normal attached embryo but the protein content is considerably less. In the control embryo the protein nitrogen (alcohol insoluble N) accounts for half the total nitrogen, and the α-amino nitrogen for half the alcohol-soluble nitrogen. The α-amino N is the first component to fall below the control level after excision. Subsequently the synthesis of the alcohol-soluble N and finally the protein N fails. Various concentrations of vitamins, purines and pyrimidines do not remedy the defect in the excised embryos. However the addition of the detached endosperm or arginine alone increases the protein synthesis although the growth pattern is not normal. These results suggest that the mechanism for amino acid synthesis is not complete in the young embryos and the amino acids, perhaps certain key amino acids, are normally supplied by the hydrolysis of proteins in the endosperm.

10:00. Recess.

10:15. LAWRENCE, JOHN M. and D. R. GRANT, Washington State Univ., Pullman, Wash. Nitrogen mobilization in pea seedlings. II. Free amino acids.

Pea seedlings were dissected into cotyledons and embryonic rootshoot axes at 0 and 1 day, and into cotyledons, root and shoot shafts, and root and shoot tips at 3 and 5 days. Individual free amino acids were determined by ion exchange column chromatography. No correspondence could be detected between the relative concentrations of the various free amino acids first appearing in the cotyledons and the amino acid composition of the total cotyledon proteins; nor between the free amino acid make-up of the developing root and shoot shaft tissue and that of the cotyledons. Extensive and rapid interconversion of amino acids is indicated. The amino acid composition of shoot shaft tissue is quite similar to that of the root shafts. Root and shoot tip compositions are also similar. Quantitatively, homoserine is by far the most important amino acid of all tissues of 3- and 5-day seedlings. It makes up about 12% of the dry weight of 5-day root and shoot shaft tissue. Asparagine is the next most prominent amino acid in root and shoot shafts at 3 and 5 days. In the younger cotyledons and embryonic root-shoot axes, arginine, asparagine, and glutamic acid are the most abundant, but in 3- and 5-day cotyledons, there is more γ-aminobutyric acid than any other amino acid except homoserine. Several unidentified amino acids were present in the growing parts; and a number of yet-uncharacterized peptides occur in all tissues.

10:30. BARNES, R. L. and AUBREY W. NAYLOR, Forest Service, USDA, and Duke Univ., Durham, N. C. Studies on the formation of β-alanine by pine embryos and callus tissues from aspartic acid, spermine, spermidine and pyrimidines.

Aseptically removed embryos of longleaf (*Pinus plaustris Mill.)* and slash (*P. elliottii Englm.*) pines and sterile callus tissue cultures of longleaf and sand (*P. clausa* Chapm. Vasey) pines were used to test the aspartic acid, the spermine-spermidine, and the pyrimidine theories of β-alanine biosynthesis. Incubation of pine tissues with neither aspartic acid, nor spermine, nor spermidine led to an accumulation of free β-alanine. Upon testing the hypothesis that the sequence orotic acid $\longrightarrow$ $\longrightarrow$ uridine $\longrightarrow$ $\longrightarrow$ uracil $\longrightarrow$ dihydrouracil $\longrightarrow$ N-carbamyl-β-alanine $\longrightarrow$ β-alanine is operative, it was found in each of the stepwise feeding experiments that the predicted products did appear. From the data it was concluded that pyrimidine catabolism provides an im-

portant mechanism of β-alanine synthesis in all of the pine tissues examined.

10:45. SHAFER, JOHN, JR., JAMES E. BAKER and JOHN F. THOMPSON, U.S. Plant, Soil and Nutrition Laboratory, Ithaca, N.Y. A *Chlorella* mutant lacking nitrate reductase.*

Following ultraviolet irradiation of *Chlorella pyrenoidosa,* a mutant was isolated which could not utilize nitrate nitrogen. Tests of various compounds indicated that $NaNO_2$, NH_4Cl, and members of the ornithine cycle (and closely related compounds) will serve as good nitrogen sources for the mutant. Nitrite is a good source only at low concentrations, being toxic at higher concentrations. The ability to use nitrite, but not nitrate, suggested an abnormal nitrate reductase system. This was investigated with cell-free extracts, prepared by grinding cells with glass beads and K_2HPO_4 solution in a Virtis homogenizer. Wild-type cells yielded extracts containing active nitrate reductase, whereas the same procedure failed to produce an active extract from mutant cells. The protein contents of wild-type and mutant extracts were determined, as well as the enzyme activity of the mutant extract and of serial dilutions of the wild-type extract. Calculations based on these data show that the mutant has less than 0.5% of the nitrate reductase activity of the wild-type, on a protein basis. A test for inhibitor in the mutant extract showed that there was none. On the basis of all available data, it was concluded that the mutant lacks an active nitrate reductase.

11:00. VAN NOORT, G., W. HUDSON and S. G. WILDMAN, Univ. of California, Los Angeles, Calif. Enzymatic and antigenic properties of fraction I protein from spinach and tobacco leaves.

Fraction I protein isolated from spinach and tobacco leaves by differential centrifugation was compared with that obtained by using polydextran (Sephadex G-75) and N,N-diethylaminoethylcellulose column chromatography. This protein fraction contains the enzymes phosphoriboisomerase, phosphopentokinase and carboxydismutase. The substrate concentrations, cofactor requirements and the products of the enzyme reactions were determined. The major product was 3-phosphoglyceric acid. Antiserum to Fraction I protein was produced in rabbits. The antiserum reacts with Fraction I protein quantitatively in a precipitin analysis. The action of the antiserum with the enzymatic reaction is under investigation.

11:15. BULA, R. J. and P. F. LOW, Purdue Univ. and USDA, Lafayette, Ind. Changes in the partial specific volume of alfalfa root cytoplasmic proteins associated with the development of cold resistance.

Densities of alfalfa root cytoplasmic protein suspensions of a winter hardy and non-winter hardy alfalfa variety were compared using extracts of field grown roots sampled in October, November, and January. The partial specific volume of the proteins in the suspensions increased concurrently with exposure to sub-freezing temperatures and the development of cold resistance. This increase was most pronounced in the winter hardy variety. Small changes in the partial specific volume of the water in the suspensions with increasing protein concentration were noted in extracts made from the hardy variety sampled in November and January but were not apparent in the non-hardy variety.

These partial specific volume changes of the protein and water suggest a structure different from that of normal free water around the protein molecules which in turn could effect the ability of the cell to withstand sub-freezing temperatures.

11:30. MILLAR, ELNORA, MARION BOURKE and C. A. PRICE, Rutgers—The State University, New Brunswick, N.J. Failure of protein synthesis in zinc deficiency.

In seeking to identify the roles of zinc in growth we have focused on protein synthesis as a primary growth parameter. A plausible hypothesis to account for an observed failure of protein synthesis in zinc deficiency is that whereas oxidation of exogenous substrates continues in zinc deficiency, although at a decreased rate (cf. Millar and Price, Plant Physiology 35 xxiii 1960), assimilation of carbon into protein is blocked.

To test this hypothesis, the time course of synthesis of proteins, amino acids, and carbohydrates was examined. *Euglena gracilis* (Klebs), z strain, was grown on an otherwise complete medium in the dark with ethanol as the principal carbon source and with concentrations of zinc either sufficient (10^{-5}M) or limiting (4×10^{-7}M) to growth. In zinc sufficiency the protein content of the cultures followed kinetics of binary fission ($P_t = P_o e^{\alpha_t}$) over a 15-fold range of protein concentrations. The rate of protein synthesis in the zinc deficient cells was at first indistinguishable from the controls. After several days the specific reaction rate, α, decreased progressively toward zero. Subsequently NPN (TCA-soluble nitrogen) of the deficient cells decreased below the control rate.

Therefore, failure of assimilation of exogenous substrates into the NPN-fraction could not account for the failure of protein synthesis. Lesions in the synthesis of individual amino acids, however, have not been excluded.

11:45. ABBADI, S. and A. WALLACE, Univ. of California, Los Angeles, Calif. Amino acid and metal effects on $C^{14}O_2$ fixation with phosphoenolpyruvate and transamination of the oxaloacetate product in preparations from leaves and fruits.

Preparations from citrus leaves and fruits fix CO_2 with phosphoenolpyruvate as substrate (*Proc. Amer. Soc. Hort. Sci.* 74:348-357, 1959). With leaf material (this buffer, pH 7.5) in the presence of glutamate and aspartate (20 μM/ml reaction mixture) and with Mg as the metal activator, a 50% and a 70% increase in $C^{14}O_2$ fixation was obtained for the amino acids respectively. With Mn as the metal activator the increase was 90% and 0% respectively. With preparations from fruit vesicles the two amino acids (4 μM/ml reaction mixture) respectively resulted in 20% and 10% increase for Mg and 70% and 20% for Mn. In both leaves and fruits and for both metals, transamination and aspartate-exchange reactions were demonstrated. This corresponds with results of Hiller and Walker (*Biochem. J.* 78:56-60, 1961). About 70-90% of the C^{14}-oxaloacetate was transaminated to C^{14}-amino acids. Passing the material through sephadex to remove low-molecular weight materials or removing mitochondria resulted in no changes in results. The effect of aspartate and glutamate in increasing $C^{14}O_2$ fixation may be analogous to

that of cyanide which stabilizes C^{14} -oxaloacetate by formation of the cyanohydrin (*Plant Physiol.* 36:118-120, 1961). Studies are in progress to determine if the differential effect of Mg and Mn with glutamate and aspartate is on the transamination reactions or on $C^{14}O_2$ fixation in the presence of the amino acids. The presence of the amino acids appeared to increase the need for Mg or Mn. The amino acids resulted also in increased $C^{14}O_2$ fixation with ribose-5-phosphate as substrate.

12:00. CLEMENTS, R. L. and HARRY V. LELAND, Riverside, Calif. An ion exchange study of seasonal changes in the amino acids of Valencia orange juice.

The ion-exchange procedure of Moore, Spackman, and Stein was employed in a study of quantitative variations of amino acids in juice of the Valencia orange during growth, maturation, and senescence. Fruit was sampled at monthly intervals over a period of seventeen months (August, 1959, to December, 1960), and the clarified juice was added directly to the columns. Paper chromatography was utilized for qualitative analysis. Over twenty different ninhydrin-positive compounds were detected, and fifteen were measured quantitatively over the season. On a molar basis, asparagine predominated in the early fruit, but declined to a steady level. Aspartic acid, serine, glutamic acid, and ammonia, together with asparagine, accounted for most of the amino nitrogen in the green fruit, but contributed relatively little after the onset of maturity. Proline was present in traces in the early fruit, but increased steadily during the season and predominated in the mature and late fruit. Arginine and γ-aminobutyric acid also showed a steady increase, but never approached the concentration of proline. Throughout the season, nitrogen calculated from the amino acids accounted for approximately 75 per cent of the Kjeldahl nitrogen in the clarified juice.

SESSION C3. Room 201, Civil Engineering

GROWTH I

C. A. Swanson, *presiding*

8:25. Announcements.

8:30. LYON, CHARLES J., Dartmouth College, Hanover, N.H. The relative strength of branch epinasty.

When young potted plants with plagiotropic branches are placed on horizontal clinostats under natural light and rotated slowly about the axes of the plants, the growth zones of the branches show epinastic curvatures. They appear to be due to the absence of normal, lateral, geotropic transport of auxins. The strength of this imbalance in growth regulation has been estimated by determining the minimum angular position between the horizontal and vertical positions to which the axis of the clinostat and plant must be moved in order to prevent growth curvatures in the branches. From such tests with six species of plants it has been found that the minimum angle with the horizontal position is 27°. Smaller angles allow epinastic curvatures at rates which increase with the decrease in the effective component of gravity. The strength of the imbalance can therefore be compared with that of a force with an acceleration of 445 cm/sec^2, as computed from the sine of 27° times 980 cm/sec^2. This imbalance in growth is interpreted as reflecting essentially the same inequality of transport of growth regulators on the dorsal and ventral sides of branches in all six species of plants tested. The imbalance is even greater toward the dorsal side when the clinostat tests are carried out in darkness, in agreement with the action of light on the redistribution of auxins in stem tissues.

8:45. SCHRANK, A. R., Univ. of Texas, Austin 12, Texas. Growth and geotropic curvature of Avena coleoptiles in the presence of N-1-naphthylphyhalamic acid.

The effects of 1×10^{-7}M to 1×10^{-4}M NP on the growth of 5 mm floating *Avena sativa L.* sections, on elongation of 15 mm apical segments and on geotropism and phototropism of apical segments were measured. Growth of floating sections was increased by 1×10^{-6} and 1×10^{-5}M NP, but only in the presence of exogenous IAA. One $\times 10^{-4}$M NP was slightly inhibitory to growth of floating sections in the presence or absence of IAA. One $\times 10^{-5}$M NP induced a slight stimulation of elongation of 15 mm apical coleoptile segments for the entire 24 hour growth period. Higher concentrations of NP (1×10^{-4}M) caused increased elongation during the first 6 hours of exposure and inhibition for longer growth periods. Two concentrations of NP (1×10^{-6} and 1×10^{-7}M) had no effect on apical segment elongation. Both 1×10^{-4}M and 1×10^{-5}M NP were inhibitory to negative geotropic curvature of 15 mm apical Avena segments, while 1×10^{-6}M and 1×10^{-7}M caused slight stimulation of bending for stimulation times up to 90 minutes. Experiments utilizing klinostatic development time subsequent to geotropic stimulation, indicated that NP treated coleoptiles attained smaller curvatures than untreated segments. Positive phototropism, in response to 270 meter-candle-seconds of unilateral illumination, also was inhibited by 1×10^{-4}M NP. Ninety minutes after application of the light, all other concentrations of NP induced stimulation of phototropic bending. For 1×10^{-6}M and 1×10^{-7}M the stimulatory effect not evident 180 minutes after exposure to light. These results were interpreted to indicate that NP does not function as a typical auxin and that NP mediated inhibition of geotropic bending during the period of stimulation in the horizontal position.

9:00. FREEBAIRN, H. T., United Fruit Co., La Lima, Honduras. Geotropic response and the movement of growth materials causing curvature of banana fruit.

Results have led us to believe that the orientation to gravity of young banana fingers determines the curvature of the fingers and the ultimate shape of the stems of fruit. By maintaining a stem of fruit in a vertical position on the plant a completely reversed curvature of the fingers was obtained. This entire curvature response occurred during the first 3 to 4 weeks after shooting with the greatest effect being produced by the stem orientation during the first 1-½ weeks.

By severing certain sections of vascular tissue in fingers, it was found that the materials responsible for curvature moved through the pulp in a spiral manner corresponding to the vascular elements present. These growth materials did not seem to be produced by the ovaries since curvature of male fingers was also found. The movement of growth materials from one side of the banana finger to the other best explains the results.

9:15. FINN, J. C., Jr. and O. D. R. BROWN, North American Aviation, Downey, Calif. Plagiogeotropism in petioles of *Xanthium*.

Orientation of ½-expanded to mature-leaf petioles of Xanthium was studied in respect to gravity, light and other plant organs. Less than 1 x gravity (lg) accelerations were simulated by clinostats adjustable between the vertical and horizontal, and by radially aligned clinostats fixed horizontally on the periphery of a 13-foot centrifuge. Orientation was determined by the angle between the stem and a line tangent to the petiole at the point of leaf attachment.

Petioles of intact plants assumed different reproducible angles of orientation at different accelerations between Og and lg in low light.

Exposure to less than lg in light expanded the angle of orientation whether the petiole was associated with the blade and/or plant, or was detached-debladed; in dark, attachment to the plant (but not blade) was required. At lg in light, following a low-g treatment, the angle contracted if the petiole was attached to plant and blade, plant alone, or was detached-debladed. No contraction occurred if the petiole was attached only to the blade.

Further, preliminary data suggest that neither the main stem apex nor lateral buds are required for petiole orientation.

9:30. HESS, CHARLES E., Purdue Univ., Lafayette, Ind. The mung bean bioassay for the detection of root promoting substances.

A bioassay for the detection of substances capable of stimulating root initiation, other than indoleacetic acid (IAA), has been developed. Seedlings of mung bean (*Phaseolus aureus*, Roxb.) are grown either in a physiological dark room or in the light. The seedlings are ready for use in 5 days from the time the seed is planted in the dark room and in 10 days when planted in the light. In the dark room experiments, the seedlings are decapitated 24 hours before cuttings are to be taken. A cutting consists of a 7 cm. section of the etiolated hypocotyl, measuring from the point of decapitation. Cuttings taken from the light grown seedlings consist of the primary leaves, the epicotyl, and 3 cm. of the hypocotyl. The cotyledons are removed. Root initiation begins within 48 hours in both the etiolated and light grown cuttings, and the roots are sufficiently long to count in 6 days. The number of roots initiated on treated cuttings in comparison to the controls is used as the measure of biological activity.

The unique features of the bioassay are small seed size which makes possible the growing of a large population of uniform seedlings in a small area, rapid germination and growth of seedlings, hypocotyls of small diameter which facilitates the bioassay of chromatogram sections, and rapid rooting. However, the most important feature of the mung bean bioassay is that there is only a slight response to the application of IAA or carbohydrates in the form of sucrose or fructose. Therefore, the bioassay can be used to detect root promoting substances other than IAA or carbohydrates.

9:45. HESS, CHARLES E., Purdue Univ., Lafayette, Ind. A comparative analysis of root initiation in easy- and difficult-to-root cuttings.

Alcoholic extracts have been made from juvenile and mature forms of *Hedera helix*, L. and red and white flowering forms of *Hibiscus Rosa-sinensis*, L. The juvenile *Hedera* and the red *Hibiscus* are easy to root; the mature *Hedera* and the white *Hibiscus* are difficult to root. The extracts were partialy purified by paper chromatography. The chromatograms were assayed for biological activity with the *Avena* coleoptile and first internode straight growth tests. Although seasonal differences in auxin and inhibitor content were large, there was little difference between the juvenile and mature *Hedera helix* tissue, regardless of the season. However, when the chromatograms were assayed with the mung bean rooting test, four areas on the juvenile *Hedera* chromatograms contained substances which stimulated root initiation. Corresponding chromatograms from mature *Hedera* either lacked the active areas or contained smaller quantities of the root promoting substances. Similar results were obtained with the red and white flowering forms of *Hibiscus*.

10:00. Recess.

10:15. KETELLAPPER, H. J. and J. BONNER, California Institute of Technology, Pasadena, Calif. The chemical basis of temperature responses in plants.

Plants respond to adverse climatic conditions with reduced dry matter accumulation. An attempt has been made to protect plants against non-optimal temperatures by application of substances which play important roles in the growth and development of plants.

Plants were grown at different temperature, including the optimal temperature, and sprayed regularly with a mixture of B vitamins, vitamin C, caseinhydrolysate, sucrose or ribosides. Application of 10% sucrose solution to pea plants grown in artificial light at 23°C day temperature, 17°C night temperature (23/17°C) caused a 56% increase in dry weight, which made the dry weight equal to that of plants grown in optimal conditions (17/17°C).

Further experiments were carried out in greenhouses. Increases in dry weight up to 40% were obtained by application of vitamin B or ribosides to pea plants grown in high temperature (30/23°C.) Application of the vitamin B mixture or thiamin to Cosmos in cool temperatures (17/10 or 20/14°C) produced a 30% increase in dry weight. Vitamin C was effective at 20/14 and 23/17°C. Soaking of tomato seeds in nicotinic acid (100 mg/1) for 24 hours gave a 30% increase in dry weight. The treatments mentioned had no significant effect at the optimal temperature.

A partial prevention of the reduction in dry weight caused by unfavorable temperature is possible by application of essential metabolites. This establishes the basic concept that environmental factors influence growth because they affect specific biochemical events.

10:30. STOTZKY, GUENTHER, ELSIE A. COX and ROGER D. GOOS, United Fruit Co., Norwood, Mass. Alternating temperature requirement for the germination of *Musa balbisiana* Colla seeds.*

Seeds of the banana, *Musa balbisiana* Colla, do not germinate at constant temperatures, even when incubated for more than 6 months at temperatures ranging from 18 to 35° C, but

germination can be induced by alternating temperatures. Maximum germination is influenced not only by the high and low temperatures, but also by the temperature differentials. The apparent optimum low temperature is 18° C and the apparent optimum high is 32° C; the optimum differential at various high and low temperatures ranges from 8 to 20° C. Five to six days of alternating temperatures will induce germination, but a longer period is required for normal seedling development and high germination percentages. The time required for initiation of germination is shortened as the high temperature is increased. Mechanically scarified seeds also require alternating temperatures for germination, but the temperature differentials required do not appear to be as great as for intact seeds. The excised embryo develops normally at constant temperature.

10:45. KUMAR, SUSHIL, ALBERT C. HILDEBRANDT and ALBERT J. RIKER, Univ. of Wisconsin, Madison, Wis. Effects of temperature on growth and tuberization in potato plants *in vitro*.

Potato plants were grown from stem sections, sprouts, seeds and embryos *in vitro* on nutrient media. Growth was compared on T-medium, a basal mineral salts-sucrose medium; or C-medium, which is T-medium with coconut milk (CM) and naphthaleneacetic acid (NAA); or D-medium, which is C-medium with 2,4-dichlorophenoxyacetic acid (2,4-D). In all cases D-medium induced only callus formation and failed to promote roots and stems. Plant growth was good on the basal T-medium but was better on C-medium. Some embryos on C-medium grew directly into seedlings while others formed only callus. However, callus tissues left long enough on C-medium usually developed leaves and roots. Tubers were formed on some plants on all media. When such potato plants were incubated *in vitro* for 8 hours of light in 16°, 20° and 24° C greenhouses and for 16 hours in the dark between 6° and 11° C the tubers produced decreased progressively in number at 16°, 20° and 24° C. Plants kept continually in a greenhouse at 25°C produced the fewest tubers. Plants from seeds grew better than those from embryos. Plants at 24°C had relatively slender stems and small leaves. Plants at 16° and 20°C appeared alike.

11:00. POSNER, H. B., Yale Univ., New Haven, Conn. Effects of x-irradiation on "handedness" of frond production in *Lemna perpusilla*, 6746.

Most species of *Lemna* produce daughter fronds in an asymmetric, alternating order which is consistent within a clone. *L. perpusilla*, strain 6746, is left-handed; i.e., the first daughter frond is formed in the meristematic pocket on the left side of the mother frond. If flowering occurs, a single flower is produced in the pocket on the right side. Spontaneously shifted (right-handed) daughters are produced rarely, and then usually by sensecent mother fronds or by fronds produced late in the life of a mother frond. A shifted daughter produced under flowering conditions, forms a single flower in the left-pocket or a flower in each pocket.

X-rays increase both the frequency of shift-producing mother fronds and the number of shifted daughters per mother. Irradiated mother fronds produce shifted daughters earlier than do nonirradiated controls. Under appropriate conditions, x-irradiation induces shifts without affecting growth. Heat and cold shocks inhibit growth but do not cause shifts. To date, the only agents, other than x-rays, found to induce shifts are 2,3,5-triiodobenzoic acid and parachlorophenoxyisobutyric acid.

A study of factors that might modify or mimic the x-ray effects is now in progress.

11:15. GRUEN, H. E., Harvard Univ., Cambridge 38, Mass. Growth regulation in mushrooms.

Detailed studies of elongation and of the increase in cap dimensions were carried out on carpophores of *Agaricus bisporus* grown in small compost flats at 15°C under constant illumination. Carpophores were decapitated at different lengths and stipe growth was followed with time. While decapitation at a very early stage of development (around 1 cm or less) results in total cessation of growth, older stipes continue elongation at a reduced rate, the extent of this 'residual growth' depending on the stage of development at the time of operation. The average total residual growth reaches a maximum in carpophores decapitated at the 4-5 cm stage and elongation continues for an average of 3 ± 1 days in this case. Despite the phenomenon of residual growth, comparison with intact sporophores and sporophores without certain portions of the cap demonstrates that the cap is required for normal stipe elongation. There is no regeneration of a physiological apex in decapitated stipes. — Many measurements following various selective operations on the cap showed that the gills play a predominant role in stipe elongation but do not alone fully account for normal growth. The cap deprived of gills reveals the existence of residual growth in a manner resembling decapitated stipes. The presence of gills greatly promotes cap expansion. The results of agar diffusion tests will be presented in regard to straight growth and curvature of stipes. Examples of photographic analysis of curvature development with time will be shown. — Despite superficial analogies with the behavior of the *Avena coleoptile* the mechanism of growth regulation in *Agaricus* carpophores is probably quite different.

11:30. GUERRERO, D. and W. E. LOOMIS, Iowa State Univ., Ames, Iowa. A resistance to storage molds in vigorously viable corn grain.

The germinability and vigor of seed-quality corn was reduced stepwise by heating with 10 per cent moisture for 2 to 24 days at 50°C. When grains of varying viability were inoculated with mixed cultures of storage molds, moistened to 20 to 25 per cent moisture and incubated at 30°C, there was an inverse relation between viability and mold development.

When damaged but not dead grains were marked and incubated in a mixture with vigorously viable grain, a heavy mat of molds grew through the culture. After one week the grains were separated and their germination tested. The heat-damaged kernels were now all dead while the controls showed little or no injury. Inoculated, injured corn heated 15°C in 4 days in thermos bottles while the controls showed only 8°C of heating.

Mechanical injury of the grain increased mold development when the endosperm was injured but not when the germ was cut with minimum killing of cells. Dyes penetrated readily around the pedicel of both control and heated grain.

The results indicate that typical storage molds are primarily saprophytic, developing on dead cells, and spreading to other tissues only after the natural resistance of these cells has been reduced by aging or by the action (excretions?) of molds growing on dead cells.

11:45. HECK, W. W. and E. G. PIRES, Texas A. and M. College, College Station, Texas. The growth of plants fumigated with saturated and unsaturated hydrocarbon gases.

Industrialization, continued increases in motor vehicle travel, and development of atomic devices is focusing attention on the problem of air pollution. Many hydrocarbon compounds contribute significantly to the overall problem of pollution, especially in metropolitan areas. However, most pollution studies have involved the whole complex of atmospheric hydrocarbon gases with little attempt to distinguish specific toxicants. The present study was undertaken to determine the specific effects of low molecular weight hydrocarbon gases on plant growth and development. A series of ten small (18 x 23 x 37 inch), gas tight, plant growth chambers has been constructed for growing plants in various gaseous atmospheres. Light, temperature, air flow and gas flow are carefully controlled in these chambers. Chamber construction, lighting, air flow and gas injection will be briefly discussed. These chambers have been used to study the effects of thirteen saturated and unsaturated hydrocarbon gases on plant growth. Initial experiments were run for one week at 1000 ppm of gas in air (volume). Five of the test gases produced definite injury symptoms at this concentration. These were re-tested at 100 and 10 ppm. Cotton, soybean, squash, corn and cowpea were the test plants used in all fumigation studies. Quantitative plant growth data and typical injury symptoms produced by each gas on each plant species will be discussed.

SESSION C4. Room 318, Memorial Center

WATER RELATIONS

G. W. Todd, *presiding*

8:25. Announcements.

8:30. BERNSTEIN, LEON, U.S. Salinity Laboratory, Riverside, Calif. Osmotic adjustment of plants to saline media.*

The osmotic pressure (OP) of roots, and of the aboveground parts, as well, of cotton, pepper, and bean plants, adjusts completely to as wide a range of OP in the root medium (salinity) as would permit any growth. Previous findings of only restricted OP adjustment in roots are shown to be caused by dilutions of cell cap by endosmosis during rinsing and to admixture with free-space solution during expression of the sap. Even young tissues such as partially expanded leaves already exhibit the full degree of osmotic pressure adjustment. Several mechanisms for osmotic growth inhibition are considered in light of the demonstrated capacity for internal osmotic adjustment. The osmotic adjustment itself is suggested as the most likely growth-limiting factor. Salt accumulation in the tissues contributes to a variable degree to OP adjustment depending on the plant organ, its age and capacity for salt accumulation and the absorbability of the salt in the medium. An increase in monovalent ions at the expense of polyvalent ones (especially the substitution of chloride for organic acids) can be a major factor in increasing the OP. In some experiments (bean), all plant parts adjusted to an increase of 1 atm OP in less than half a day; in others (pepper), several days were required.

8:45. BENDA, GERD, Univ. of Notre Dame, Notre Dame, Ind. Air bubbles and living trichomes.*

This report concerns observations on the protoplasm of cells containing air bubbles. Techniques for the establishment of an intracellular interface of air and protoplasm are described, the behaivor of the exposed protoplasm is examined, and the rate of solution of the intracellular air is considered. The trichomes of the stem of *Nicotiana miersii* Remy consist of a uniseriate row of long cylindrical cells. When a cut is made into cells which are not fully turgid, and the cut is near either end of the cell, then air may enter as a column or as one or more bubbles. An air bubble, enclosed within the cell vacuole, may be caused to move away from the wound by tilting the cell. Such a cell mounted in 0.4M sucrose may show strands of protoplasm streaming toward and away from the interface where plastids and other inclusions appear to have become fixed. If the air bubble in its movement stops near the nucleus, then the nucleus may be seen to spread itself upon the bubble surface. Cells mounted in sucrose after wounding usually remain viable for less than a day. A single bubble may decrease in volume at a constant rate, or the bubble may maintain itself at constant volume for some time. In living cells the bubbles usually disappear in a few hours, in dead cells they may persist for days. The surface of the bubble depends on the state of protoplasm through which the bubble has moved. A severely injured or dying cell shows bubbles coated with coagulated protoplasm whereas a living cell may show large areas of the bubble to be "clean" at a thousandfold magnification. It is the conclusion of this study that, contrary to commonly accepted views, protoplasm in contact with air may survive for at least some hours visibly undamaged.

9:00. BRUN, WILLIAM A., United Fruit Co., Norwood, Mass. Photosynthesis and transpiration of banana leaves as affected by severing the vascular system.

Continuous measurements of apparent photosynthesis, transpiration and leaf temperature were made on the lower surfaces of young attached banana leaves. At a specified time during the period of measurement the vascular supply to the leaf was interrupted by cutting either on the blade, on the petiole, or at the base of the pseudostem.

Irrespective of where the cut was placed, it always caused an immediate stimulation of transpiration and apparent photosynthesis, and a drop in leaf temperature. In young plants with thin pseudostems, the stimulation was transient and the rates of the two processes soon fell to zero.

In more mature plants with thick succulent pseudostems, the initial stimulation became more prolonged as more tissue was included between the cut and the leaf under measurement. When the cut was placed at the base of such a thick pseudostem, the initial stimulation gave way to a series of repeated fluctuations in the rate of CO_2 uptake and transpiration, which lasted for about 5 hours.

Throughout the experiments, the curves representing the time courses of apparent photosynthesis and transpiration resembled each other very closely. Leaf temperatures always fluctuated inversely with transpiration rates.

9:15. YOO, B. Y. and G. W. TODD, Oklahoma State Univ., Stillwater, Okla. Nucleic acid and enzymatic changes in desiccated wheat leaves.

Detached Cheyenne wheat leaves were dried for various lengths of time up to 40 hours over $CaCl_2$. The amount of soluble RNA rapidly decreased with a decrease in water content and in leaves containing less than 20% of the original water the RNA was 42% of the initial value. A similar trend was noted for soluble protein. The activities of the enzymes peptidase, proteinase, peroxidase, succinic dehydrogenase and acid phosphatase were also followed as the leaves were desiccated. The activity of peroxidase, peptidase and succinic dehydrogenase did not change appreciably over a wide range of water content. Proteinase activity decreased with a decrease in water content and was almost nil at 30% of the original water content. Acid phosphatase remained constant to about 60% water content and then completely disappeared with further desiccation.

When leaves were detached but kept in a saturated atmosphere for 40 hours the enzyme changes were somewhat different. The enzyme peptidase and acid phosphatase increased over the control, peroxidase and succinic dehydrogenase decreased slightly and proteinase activity reached a low value.

These results indicate that desiccation leads to a different pattern of enzyme destruction than does detachment without desiccation.

9:30. RANEY, FRANKLIN, Univ. of California, Davis, Calif. Certain aspects of water movement in sunflower plants using tritium as a tracer.

Afflux of tritiated water was measured at various times in intact and segmented sunflower plants of different ages grown in aerated nutrient solution in a constant environment chamber. Segments of sacrificed plants were frozen with dry ice. Aliquots of tissue water captured by a lyophilization technique were assayed for radioactivity using a liquid scintillation beta spectrometer.

Root tissue of sunflower plants attained external THO concentrations. Equilibration times differed markedly depending upon transpiration rates. In detached entire root systems and in root systems attached to the shoot in the dark equilibration half-time was one-half minute. In roots attached to the intact shoot in the light equilibration half-time was at least half an hour. An explanatory hypothesis is offered.

9:45. EHLIG, C. F., U.S. Salinity Laboratory, Riverside, Calif. Measurement of the energy status of water in plants with a thermocouple psychrometer.

A thermocouple psychrometer provided a simple and relatively rapid method for measuring diffusion pressure deficit and osmotic pressure in plant leaves. Leaves or sections of leaves were placed in a sample chamber held at a temperature of 25.000 ± 0.003°C. The relative humidity in the sample chamber was determined with a wet and dry junction thermocouple devised by L. A. Richards and Gen Ogata. The diffusion pressure deficit and osmotic pressure in the leaves of trefoil, pepper and cotton were correlated with the relative water content of the leaves.

10:00. Recess.

10:15. TURRELL, F. M., S. W. AUSTIN and R. L. PERRY, Univ. of California, Riverside and Los Angeles, Calif. Nocturnal thermal exchange of citrus leaves in warm and freezing temperatures.

Rate of temperature change of cooling leaves was measured in the dark on intact greenhouse grown lemon cuttings in still air in open laboratory rooms of warm to freezing temperatures, and on four commercial varieties of citrus leaves picked from mature trees in the grove and measured in a microcosm at constant temperature using fine thermocouples threaded in the leaf laminae. Transpiration rates of detached leaves from both sources were measured in the dark through a similar temperature range. From the former total thermal conductance (h_t) was calculated, and from the latter transpiration conductance (h_e). Radiation conductance (h_r) was calculated for lemon leaves from infrared reflectance, and the free convection conductances (h_c) by the equation $h_c = h_t - (h_r + h_e)$. The free convection conductance coefficient obtained for lemon then was applied to three other varieties of citrus to obtain h_c and subsequently h_r. In warm still air (22°C) in the microcosm the conductances h_t, h_c, h_r, and h_e were 3.2, 1.2, 1.5 and 0.4 respectively; in warm still air (26.6°C) in laboratory rooms the conductances were 3.5, 1.8, 1.4, 0.3; in cold still air (−15.1°C) the conductances were 1.6, 0.7, 0.9, 0.2; in warm moving air (26.6°C) of 100 cm/sec velocity the conductances were 8.1, 6.5, 1.4 and 0.28; in cold moving air (−15.1°C) of 100 cm/sec 3.9, 3.0, 0.9 and 0.02, (cal/sec cm^2C) x 10^{-4}. Temperatures of leaves measured in the grove at night could be calculated to a mean of ±0.58°C from the derived conductances and measured micro-climatological factors.

10:30. JANES, BYRON E., Univ. of Connecticut, Storrs, Conn. Use of polyethelyne glycol as a solvent to increase the osmotic pressure of nutrient solutions in studies on the physiology of water in plants.

The effect of altering osmotic pressure of nutrient solution with polyethelyne glycol (P.E.G.) of different size molecules on transpiration, growth and absorption of P.E.G. by tomato and celery plants was determined. The plants were subjected to different environmental condition by growing them in open, in greenhouse or in a controlled environment facility. A rapid change from low to high osmotic pressure of solution injured roots and tops of plants especially if they were subjected to high moisture stress. The osmotic pressure of nutrient solution of tomato plants growing at 26°C, 60% relative humidity and 2000 foot candles of light was gradually increased with P.E.G. of different molecular weights. Plants growing in solution of P.E.G. with molecular weights of 400, 600, 1000, 1540, and 4000 appeared normal with no indication of injury from the chemicals. Roots of plants growing in solution with P.E.G. of 6000 molecular weights were injured. A reduction in rate of transpiration was first observed when the osmotic pressure of the nutrient solution reached 2 atmospheres. As the osmotic pressure was increased the rate of transpiration decreased. Tomato plants growing in standard Hoaglands solution transpired 6 times as much water as plants growing in Hoaglands solution with enough P.E.G. 1540 to give an osmotic pressure of 14.4 atmospheres. Increasing the osmotic pressure of the nutrient solution reduced the rate of growth of plants. There was no increase in weight of plants in solu-

tions above 11. atmospheres osmotic pressure. Measurable amounts of P.E.G. were not found in the plants until the osmotic pressure was above 5 atmospheres. After 9 days in a P.E.G. 1540 solution with a final osmotic pressure of 14.4 atmospheres, the P.E.G. content of the plants was approximately 0.1% of fresh weight.

10:45. WOOLLEY, JOSEPH T., USDA, Univ. of Illinois, Urbana, Ill. Soil-applied hexadecanol as a transpiration-suppressant.

Laboratory studies were made of the effect of soil-applied hexadecanol on transpiration of corn and soybeans and on evaporation from soil. One-half percent hexadecanol in soil (w/w) reduced growth of corn and soybeans, while smaller amounts of hexadecanol had no effect on water requirement. Under some conditions hexadecanol decreased water loss from soil surface.

TUESDAY AFTERNOON

SESSION D1. Loeb Playhouse, Memorial Center

***Symposium:* FRONTIERS IN PLANT PHYSIOLOGY**

A. W. Naylor, *presiding*

1:30. LANG, ANTON, California Institute of Technology, Pasadena, Calif. Some aspects of plant growth controlled by environment.

ZILL, L. P., Research Institute for Advanced Studies, Baltimore, Md. Lipids of photosynthetic tissue.

NAYLOR, AUBREY W., Duke Univ., Durham, N.C. Some aspects of plant metabolism associated with arginine, ornithine and citruline interconversions.

SESSION D2. Loeb Playhouse, Memorial Center

GENERAL BUSINESS MEETING OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Immediately following symposium.

WEDNESDAY, AUGUST 30

WEDNESDAY MORNING

CONCURRENT SESSIONS E1, E2, E3, E4

SESSION E1. Room 318, Memorial Center

PHOTOSYNTHESIS III

G. Krotkov, *presiding*

8:25. Announcements.

8:30. COBB, HOWELL D. and JACK MYERS, Univ. of Texas, Austin, Texas. Interrelationships between nitrogen fixation and photosynthesis in a blue-green alga.

We are in search for conditions under which the relationships between carbon dioxide reduction and nitrogen fixation in a blue-green alga can best be demonstrated. Our experimental material is *Anabaena cylindrica* grown in continuous culture in absence of any fixed nitrogen. Our initial approach is to develop conditions of N-deficiency in hope that subsequent exposure to N_2 will result in extra-rapid N-assimilation as observed in *Chlorella* with other N-sources.

An illuminated suspension under 0.5% CO_2 in argon shows continued increase in dry weight with attendant decrease in cell-nitrogen content. Subsequent incubation of such N-deficient cells in N_2, N_2 plus O_2, or in N_2 plus CO_2 increases the cell nitrogen content. Manometric studies of N-deficient cells in N_2 are interpreted as showing a small N_2 uptake accompanied by O_2 evolution. Current work is designated to determine conditions of cell history and manometry under which this effect can best be observed.

8:45. SOROKIN, C., Univ. of Maryland, College Park, Md. Fluctuations in photosynthetic activity in synchronously grown algal cells.

Studies on synchronized algal suspensions indicate that photosynthetic activity generally does not remain constant in the course of one experiment but undergoes changes whose direction, rate, extent, timing, and dependence on external conditions vary with the progress in cell development. Under given experimental conditions these variations are, presumably, due to changes in the size of the active part of the photosynthetic mechanism.

In younger cells, suspended in nitrogen-free medium, photosynthetic activity generally increases with time, indicating a prevalence of the constructive over degradation processes. In older cells the activity declines in the course of an experiment, indicating a preponderance of degradation over constructive processes. In cells of intermediate developmental stages, suspended in phosphate buffer, the activity may first decline and later recover to the original level or even to a higher level than that of the initial rate.

This sequence of the downward and the upward trends in photosynthetic activity indicates that in cells of intermediate age both degragation and constructive processes have a measurable expression. The chances of identifying the site of the turnover with photosynthetic pigments, enzymatic part of the

photosynthetic mechanism, or with enzyme systems to which photosynthesis is connected by coupled reactions, are discussed.

9:00. MILNER, H. W., and W. M. HIESEY, Carnegie Institution of Washington, Stanford, Calif. Photosynthetic rate and light saturation from 0 to 50° in altitudinal races of *Mimulus*.

The photosynthetic rates and light saturation requirements of several distinct climatic races of *Mimulus cardinalis* respond differently to changes in temperature. The range covered was 0 to 50°. Measurements were made on races whose native habitats range from sea level to 7800 feet altitude.

9:15. WICKLIFF, J. L. and S. ARONOFF, Iowa State Univ., Ames, Iowa. Quantitative measurement of leaf chlorophylls by spectrophotometry of their pheophytins in aqueous alcoholic extracts.

Determinations of leaf chlorophylls were made as their pheophytins in acidified 80% equeous ethanolic extracts. The solutions resulting from two different methods of extraction were analyzed for their pigment content with three different commercial instruments—a colorimeter and two spectrophotometers. In one extraction procedure, the pigments from dried leaf samples were extracted by grinding with 80% aqueous ethanol, followed by centrifugation; the supernatant pigment solution was made 0.01 M in HCl prior to anaylsis. In the second procedure, the pigments were extracted from frozen leaf samples with simmering 80% aqueous ethanol, followed by decantation and acidification prior to analysis. The pheophytin in the extract solutions thus prepared was measured as total pheophytin with a Klett-Summerson photoelectric colorimeter using a red filter, or pheophytins *a* and *b* were determined individually by spectrophotometry at two wavelengths using a Beckman Model DU Spectrophotometer or a Cary Model 14 recording spectrophotometer. The precisions of these extraction and instrumental procedures will be compared. This work was supported by a grant from the National Science Foundation.

9:30. SHIROYA, TSUGIO, G. LISTER, G. KROTKOV and C. D. NELSON, Queen's Univ., Kingston, Ontario, Canada. The nature of photosynthate in *Pinus strobus* seedlings and its translocation to roots.

Two year old *P. strobus* seedlings were enclosed separately in plexiglass chambers and illuminated for 8 hr in the presence of $C^{14}O_2$. Then each seedling was separated into its branch, stem and roots and these were extracted with ethanol, giving ethanol-soluble and -insoluble fractions. Ethanol-soluble extracts were first resolved by means of resins into three fractions containing sugars, amino acids and organic acids. Each fraction was separated further by chromatography. The radioactivity of each separated compound, as well as the total radioactivity of the ethanol-insoluble residues, were determined.

About 92% of the total radioactivity found in each seedling was in the ethanol-soluble and 8% in the ethanol-insoluble form. Seedlings, which prior to these experiments were grown in full light intensity, translocated a larger fraction of their photosynthate to roots than those grown in low light intensity. The main ethanol-soluble substance was always sucrose, constituting 74% of the ethanol-soluble fraction in the branch and 94% in the root.

9:45. KEARNEY, P. C. and N. E. TOLBERT, Michigan State Univ., East Lansing, Mich. Movement of carbon and phosphorus compounds from chloroplasts after photosynthesis.

After exposure of isolated chloroplasts to $C^{14}O_2$ for 2 to 60 minutes, the filtrate and isotonic washings were separated from the particles either by rapid vacuum filtration on celite, by centrifugation or by dialysis. The C^{14}-products in the supernatant fluid or remaining in the particulates were examined chromatographically. Of the total C^{14} fixed during 2 to 10 minutes about 50 percent was found in the supernatant fluid, and glycolate was the major product of this fraction. The compounds remaining within the plastids were the phosphate esters of the photosynthetic carbon cycle and sucrose. Glycolate-C^{14} when added to cholorplast suspensions was converted to glycine slowly in the supernatant fluid, but it was absorbed into the chloroplast at a much slower rate. However, in intact cells glycolate was rapidly metabolized: glycolate $\longrightarrow$ glyoxylate $\longrightarrow$ glycine $\longrightarrow$ serine $\longrightarrow$ glycerate $\longrightarrow$ hexoses. As examined by these techniques, ATP formed by photosynthetic phosphorylation was partially retained inside the chloroplasts and partially recovered outside of the chloroplasts. Inhibitors, such as KF and arsenate, or experiments of one hour duration caused a break down in chloroplasts as indicated by indiscriminate loss from the particles. Our working hypothesis is that a glycolate pathway from the photosynthetic carbon cycle may function for the outward transport of the newly fixed C^{14} from the chloroplasts.

10:00. HAUSCHILD, A. H. W., C. D. NELSON and G. KROTKOV, Queen's Univ., Kingston, Ontario, Canada. The effect of light quality on the products of photosynthesis in *Chlorella vulgaris*.

Suspensions of *Chlorella vulgaris* were illuminated in the presence of C^{14}-bicarbonate with red light, red plus 4% of the total energy as blue light and with blue light alone. The light intensities were adjusted so that the rate of photosynthesis (fixation of C^{14}) was the same under the different conditions of illumination. The distribution of C^{14} among the various compounds of the ethanol-soluble fraction was obtained using paper chromatography and autoradiography.

After 5 minutes photosynthesis the incorporation of C^{14} into aspartic acid was higher in the presence of supplementary blue light than in red light. At the same time the total activity of glycine-serine and glycollic acid decreased. After 30 minutes photosynthesis the incorporation of C^{14} into the amino acid and organic acid fraction increased in the presence of supplementary blue light and in blue light alone when compared to incorporation into this fraction in the red light. This increase was made up largely by an increase in the total activity of aspartic and glutamic acids.

It is concluded that the path of carbon in photosynthesis is affected by the quality of light.

10:15. TAYLOR, O. C., W. M. DUGGER Jr., M. D. THOMAS and C. R. THOMPSON, Univ. of California, Riverside, Calif. Effect of atmospheric oxidants on apparent photosynthesis in citrus trees.

The differential, infrared carbon dioxide analyser was used to determine the rate of apparent photosynthesis in citrus trees subjected to low concentrations of oxidants as air pollutants. The rate of CO_2 fixation by potted lime seedlings was significantly inhibited by exposures to ozone (0.6 ppm) for one hour without the development of leaf symptoms. This inhibition in CO_2 fixation was evident within four to five minutes after the seedlings were introduced to the ozone containing atmosphere and increased rapidly until the ozone was removed. Recovery was much more gradual, requiring several hours to return to the original rate of CO_2 fixation. Although no symptoms of leaf damage were evident after these one hour fumigations, it is known that repeated exposures to 0.6 ppm ozone for seven hours a day for seven to ten days may cause a characteristic type of necrosis in the palisade tissue.

Apparent photosynthesis in bearing orange trees was also less in ambient air containing intermittent atmospheric oxidants than in air filtered through activated carbon.

SESSION E2. Room 201, Civil Engineering

GROWTH II

E. H. Newcomb, *presiding*

8:25. Announcements.

8:30. GREEN, PAUL B. and JAMES CHEN, Univ. of Pennsylvania, Philadelphia 4, Pa. Concerning the mechanism of microfibrillar orientation in the growing cell wall of *Nitella*.*

The cylindrical growth of many plant cells appears to be based upon the predominantly transverse orientation of microfibrils in the cell wall. In many cells, including the internode of *Nitella*, this predominant orientation is maintained during elongation by the synthesis, at or near the wall inner surface, of new transversely oriented microfibrils. The oft-suggested control of this orientation by the predominant transverse stress in the cylindrical cell is apparently ruled out by the continuation of transverse synthesis in walls where the typical transverse stress is eliminated or converted to a compression. Control of the directed synthesis through simple template action of the pre-existing wall appears ruled out by the observation that poor transverse order of the wall inner surface, induced by colchicine, is not perpetuated upon removal from colchicine but is covered up by new material of good transverse order. This recovery involves a resumption of elongation by the cell. In general, transverse order in wall synthesis is associated with the predominantly axial expansion of the "stationary" cytoplasm adjacent to the wall. A simple but purely speculative model to account for this correlation would involve long molecules in the "stationary" cytoplasm which would be aligned in the axial direction by cell elongation. These long molecules would be given the property of starting new microfibrils more or less at right angles to their own long axis. A model of this general type is supported primarily by negative evidence at the time of writing. It unfortunately appears inapplicable to many other cases of oriented microfibrillar synthesis such as secondary wall formation.

8:45. FLEMION, FLORENCE, Boyce Thompson Institute for Plant Res., Inc., Yonkers, N.Y. Cytochemical studies of the developing primary cell wall in the apical shoots of normal and physiologically dwarfed peach seedlings.

Various components of the developing cell wall—pectic substances, hemicellulose, noncellulosic polysaccharides and cellulose—were studied by using a cytochemical technique developed by Dr. William A. Jensen of the University of California. Tissues killed and fixed by freeze substitution embedded in paraffin and sliced ten microns thick were differentially extracted and stained with periodic-acid Schiff's reaction for aldehydes. The first 4 mm. of the apical region of actively growing, semidormant and dormant shoots of both normal and phsysiological dwarfed seedlings and of chilled and unchilled seeds of peach were investigated. Quantitative differences were readily discernible. Irrespective of seedling development the pectic substances and especially the hemicellulose predominated in the very thin cell walls of the apical initials and of the subapical actively growing tissues. The noncellulosic polysaccharides and cellulose became much more predominant when the walls of the pith, cortex and epidermis thickened with cessation of stem elongation and its accompanying onset of dormancy. No apparent reduction in cell diameter was noted in any of these studies until after removal of the noncellulosic polysaccharides when only the cellulose remained. Small brilliantly stained starch granules, which were readily hydrolyzed by amylase, were prevalent not only in the subapical region but also present in large quantities in the various tissues of the actively growing physiological dwarfs, in dormant shoots and in the germinating seeds. In the actively growing normal shoots, it was found mainly in the starch sheath.

9:00. RAY, PETER M., Univ. of Michigan, Ann Arbor, Mich. Cell wall synthesis and cell elongation in oat coleoptile sections.

Synthesis of new cell wall in oat coleoptile sections is promoted by auxin. Inhibitors of growth fall into two classes with respect to their effect on wall synthesis: (1) Synthesis inhibited as strongly as or more strongly than growth (azide; dinitrophenol; iodoacetate; supraoptimal auxin; galactose and mannose [non-osmotic]; mannitol and carbowax 4000 [osmotic]); (2) growth inhibited much more strongly than synthesis (phenylmercuric chloride; Hg^{++}; arsenite; fluoride; Ca^{++} and Mg^{++}). With group (2), synthesis continues at about 40 percent of the control rate even when growth is completely inhibited, whereas with group (1) synthesis is essentially stopped. This indicates that the strong inhibitions obtained with group (1) are not due indirectly to inhibition of growth. Results with osmotic inhibitors suggest direct requirement for turgor pressure in cell wall synthesis.

The results support the belief that gross synthesis of cell wall is not the basis of cell enlargement, but they also suggest that a small amount of synthesis may be an indispensable part of the growth mechanism. Inhibitors of group (2) appear to act on components of the growth mechanism other than wall synthesis; they may depress synthesis secondarily.

9:15. MASCARENHAS, J. and L. MACHLIS, Univ. of California, Berkeley 4, Calif. Studies on the chemotrophic growth of pollen tubes.

A semi-quantitative bioassay for the chemical factor controlling the growth of pollen tubes of snapdragon to ovules

has been developed and will be described. A wide selection of amino acids, sugars, plant hormones, organic acids, etc., have been tested and found inactive. Positive responses are given by the stigma, style, placenta, and ovules of snapdragon but not by vegetative parts or other floral tissues.

Procedures will be described for extracting and purifying the active substance. Preliminary chemical characterization may possibly be available by the time of the meetings.

9:30. ROSEN, W. G. and L. F. KRESS, Marquette Univ., Milwaukee 3, Wis. Studies on pistil extracts causing pollen tube chemotropism in *Lilium longiflorum*.

Pollen tubes of *L. longiflorum* grow toward slices of stigma and style across the surface of a sucrose-boron-agar medium. The chemotropic factor can be extracted by mincing stigmas and styles into small fragments, covering with distilled water, and permitting the mixture to stand under refrigeration for 3-4 days. When separated from the pistil fragments and placed in wells in the agar medium, the liquid (diffusate) attracts pollen tubes growing from grains placed near the wells.

Under refrigeration, chemotropic activity of diffusate declines to zero in several days. Addition of acetone to diffusate causes formation of a gummy precipitate; chemotropic activity is retained and stabilized in the supernatant. Crude or acetone-treated diffusate retains activity following freeze-drying and reconstitution with distilled water. Chemotropic activity of acetone-treated, freeze-dried diffusate is soluble in methanol and in ethanol and insoluble in chloroform and in acetone. It is retained by a weak cation exchange resin but not by weak or strong anion exchange resins.

When pollen is germinated directly in diluted, acetone-treated diffusate, to which sucrose is added for osmotic balance, the tubes form bulges and branches at the growing tips. Positive chemotropism seems to result from an effect of a gradient of the chemotropic factor on the plasticity of the growing tip of the tube. (Supported by NSF grant G 5709.)

9:45. SIEVERT, R. C., A. C. HILDERBRANDT, R. H. BURRIS and A. J. RIKER, Univ. of Wisconsin, Madison, Wis. Growth *in vitro* of single cell clones isolated from a single cell clone.

Investigations were made to determine the variability among cells that make up the tissue produced by a single isolated cell. Callus tissue was used that originally came from the stem of the hybrid tobacco *Nicotiana tabacum* X *N. glutinosa*. Single cells were reisolated from two parent clones which had been grown from single cells. From over 300 reisolated cells 52 clones were established using the filter paper raft and microchamber methods. The cultures were grown on basal T-medium (mineral salts-sucrose) supplemented with combinations of naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, and coconut milk. Growth measurements were made as wet weights at weekly intervals. The reisolated clones appeared to maintain the differences in growth which were observed between the two parent clones. For example, in the absence of coconut milk one parent and the 22 reisolated clones from it failed to grow or grew poorly, but the other parent and the 30 reisolated clones grew relatively well. Differences in both color and consistency were observed also among the clones reisolated from a single parent.

10:00. Recess.

10:15. HEPLER, PETER K., and ELDON H. NEWCOMB, Univ. of Wisconsin, Madison, Wis. Sequential changes in fine structure during differentiation of parenchyma into xylem in wounded *Coleus* stem.

When a vascular bundle in the stem of *Coleus* is severed by a lateral incision, its continuity is re-established by a basipetal differentiation of parenchyma cells into xylem cells interior to the cut strand. This regenerative phenomenon, which has been the subject of a number of investigations and is evidently under auxin control, involves stratification of the peripheral cytoplasm of the parenchyma cells, then formation of reticulate lignified thickenings of secondary wall within the cytoplasmic strands. The experimental material used in the present investigation of the changes in fine structure occurring during this differentiation consists of internode sections which are excised, notched so as to sever the vascular bundles on one side, and cultured in petri dishes with the basal ends in 2% agar. An agar block with or without 2 mg of indoleacetic acid per liter is applied to the top of each section, and is replaced every 24 hours. Groups of cells in successive stages of differentiation are excised, fixed, embedded, and sectioned for electron microscopy. Banding of the cytoplasm in the early stages of the differentiation which results from auxin application is observed in the electron micrographs. The previously reported granulation in these bands is found to be due to the presence both of numerous mitochondria and of vesicles located in the cytoplasm near the secondary wall thickenings during development of the latter. Changes in the cytoplasmic organization and in the appearance of the reticulations of secondary wall during their deposition will be described.

10:30. MURASHIGE, T., Univ. of Hawaii, Honolulu, Hawaii. Influence of certain growth regulators on shoot formation in cultured tobacco pith.

Incorporation of gibberellic acid (GA) into the culture medium depressed the appearance of shoots under otherwise favorable conditions. Concentrations of GA above 0.5 mg/l were effective, with 5 and 10 mg/l resulting in complete suppression. Attempts to reverse the inhibition by (2-chloroethyl)-trimethylammonium chloride, 6-furfurylaminopurine, and other substances will be described.

10:45. HAGEN, GEORGE L., Institute for Cancer Research, Fox Chase, Philadelphia 11, Pa. Growth factors: abnormal growth in tobacco hybrid tissues.

Aseptically cultured amphidiploid tumor tissues of *Nicotiana glauca* x *N. langsdorffii* respond diffenently to complex biochemical additives. Acid hydrolized casein induces aberrant leafy centers—yeast extract gives meristematic centers without leaves—coconut milk produces amorphous callus like growth. Pith sections on hydrolized casein show limited growth which is leafy (first passage) and abundant growth which is leafy (second passage) while these pith sections on coconut milk have abundant growth which is amorphous on both the first and second passages. It seems probable, therefore, that hydrolized casein grown pith sections become tumorous only after a longer period of culture while coconut milk grown pith sections are induced to immediate tumor formation. The significance of this tumor inducing ability by coconut milk and

fractions thereof and the relationship of coconut milk tumor induction to normal tumor induction will be discussed.

11:00. de TOROK, DENES and THOMAS H. RODERICK, Harvard Univ., Cambridge, Mass. and Jackson Memorial Laboratory, Bar Harbor, Maine. Association between growth rate and chromosome number in a plant tissue culture.*

The study was carried out with tissues derived from a tumor of white spruce, *Picea glauca,* which is found on Mt. Desert Island, Maine, and with normal tissue from the same tree. Twelve subcultures derived from one explant of the normal tissue, and forty subcultures derived from one explant of the tumorous tissue, were studied over fourteen successive transfers. The tumor subcultures showed greater variation than the normal with respect to chromosome number, rate of cell division, and rate of growth. In both tissues, by far the greater variance in growth rate was found between different subcultures rather than in the behavior of a single subculture over successive transfers. The intra class correlation was +0.93 for the tumor subcultures and +0.67 for the normal subcultures, both significant at the 0.001 level. Highly significant correlations were found among growth rate, rate of cell division and chromosome number in both the tumor and normal tissues. The most important finding is the clear association between chromosome number and growth rate over the entire aneuploid series. As a plausible hypothesis to explain this association it is tentatively suggested that there is a cause and effect relationship from chromosome number through rate of cell division to growth rate.

11:15. SPURR, H. W., Jr., A. C. HILDEBRANDT and A. J. RIKER, Univ. of Wisconsin, Madison, Wis. Ascorbic acid oxidase and tyrosinase activities in relation to crown-gall development on tomato.

Ascorbic acid oxidase (AAO) and tyrosinase (T) activities were measured on normal, wounded and crown-gall tomato stem tissues. Upper internodes of young plants were studied at intervals up to 30 days after treatment. A growth chamber maintained 26°C at which crown-gall developed, and 32°C, at which crown-gall did not develop. O_2 utilization of homogenates was measured with an oxygen electrode. Ascorbic acid and catechol were substrates for AAO and T, respectively. Wounding increased AAO and T activities at both 26° and 32°C and activities decreased to near normal 16 days after wounding. As crown-gall developed from wound inoculations at 26° activities remained high and were characteristic of crown-gall tissue. At 32° AAO and T activities of inoculated stems followed the pattern of wounded tissue. Thus, a correlation existed between gall development and these activities. Crown-gall development was studied by inoculating plants at 32° and after 48 hours exposing them to 26°C of 0, 12, 24 or 48 hours. Sixteen days after inoculation AAO and T activities of tissues showed increased activity in proportion to the time at 26°. Thus, a direct relationship existed between the time at 26° and AAO and T activities of crown-gall. This evidence indicated that AAO and T had the same positive and negative temperature activities as crown-gall tissue.

11:30. HILDEBRANDT, A. C., HEINRICH H. HOTTINGER and A. J. RIKER, Univ. of Wisconsin, Madison, Wis. Isolation and growth *in vitro* of single cell clones from grape *Phylloxera* gall and normal stem tissues.*

Many of 1500 single cell isolations developed as independent clones in 66 to 144 days on mineral salts-sucrose-coconut milk (CM)-naphthaleneacetic acid (NAA) media without (C-medium) or with (D-medium) 2,4-dichlorophenoxyacetic acid (2,4-D). On 30 different media composed of mineral salts and sucrose, with or without CM or 2,4-D and with combinations of NAA, casein hydrolysate (CH), adenine, kinetin, inositol and 18 amino acids, the clones varied in firmness, color and growth rate. Two fast, medium and slow growing clones, respectively, from two gall and from one normal stem strains usually were constant in firmness, color, and growth rate. All 18 clones from these 3 strains grew poorly or not at all on the basal mineral salts-sucrose medium supplemented with various combinations of 18 amino acids, NAA, adenine and kinetin. Six gall clones grew well on D-medium and moderately well on C-medium. Six clones from the second gall grew well with the basal mineral salts-sucrose medium supplemented with CH, inositol and NAA, and moderately well with D- or C-media, or without CM but with CH and NAA. The six clones from normal stem tissue grew well with the C-medium, moderately well with CH, inositol and NAA and were inhibited by D-medium.

11:45. ARYA, HARISH C., ALBERT C. HILDEBRANDT and A. J. RIKER, Univ. of Wisconsin, Madison, Wis. Response of normal and *Phylloxera* gall grape callus tissues *in vitro* to concentrations of sugars.*

Growth was compared of six single cell clones of normal grape stem and of gall tissues incited by *Phylloxera vastatrix.* The clones were stable in growth rate (fast, medium fast, or slow) when grown on a basal medium with 2.0 percent sucrose and supplemented with 15.0 percent coconut milk. Concentrations from 0.06 to 4.0 percent of sucrose, dextrose, d(−)levulose, d(+)mannose, d(+)galactose, and d(+)lactose, respectively, were used. Growth was measured after six weeks as the average wet weight. Each sugar except mannose was a suitable source of carbon. Gall and normal tissue clones grew best with 0.125 percent sucrose, levulose, and galactose. With dextrose and lactose maximum yields were obtained at the 1.0 percent level. Growth of fast, medium fast, and slow growing clones was altered with the type of sugar. Gall and normal tissues were differentiated with mannose where clones of the gall tissues grew best at 0.125 percent level and normal at 1.0 percent. Sugars varied in their inhibitory influence at the 4.0 percent level. 1.0 percent dextrose, levulose, and lactose proved better than sucrose except for one gall tissue clone. At 0.125 percent, sucrose was most favorable. However, at 0.125 percent sugar levels even in cases where high yields were obtained, the physical character of the tissues changed to dry, brownish, and very friable.

SESSION E3. Room 310, Memorial Center

RESPIRATION

Harry Beevers, *presiding*

8:25. Announcements.

8:30. McGLASSON, W. B., K. S. ROWAN and HARLAN K. PRATT, Univ. of California, Davis, Calif. Effect of ethylene on immature cantaloupe fruits.

A 12-hour exposure at 1000 ppm. ethylene at 20°C, begun thirty hours after harvest, induced a climateric-like rise in carbon dioxide production by immature cantaloupe fruits (*Cucumis melo* L. var. *reticulatis*). Yellowing and softening changes similar to those observed during ripening of mature melons were noted. Analysis of high-energy phosphates, 30 hours after the beginning of the ethylene treatments, revealed that ethylene stimulated an increase in ATP and a decrease in ADP in fruits harvested 23, 28, and 32 days after anthesis, but not in fruits harvested 9 and 17 days after anthesis. These differences suggest that the metabolism of cantaloupe fruits undergoes a marked change between 17 and 23 days after anthesis. This period is closely correlated with a distinct change in the growth pattern of the fruit.

8:45. LYONS, JAMES M., W. B. McGLASSON and HARLAN K. PRATT, Univ. of California, Davis, Calif. Ethylene production, respiration and internal gas concentrations in cantaloupe fruits at various stages of maturity.

The composition of the internal atmospheres (carbon dioxide, oxygen, and ethylene) of cantaloupe fruits harvested at different stages of development were measured with sensitive gas chromatographs. Traces of ethylene appeared in melons harvested as young as seven days from anthesis. In studies of harvested mature melons, a climacteric rise in respiration was demonstrated during ripening. Coinciding with, or immediately preceding, this rise in carbon dioxide evolution was a great increase in both internal and evolved ethylene, a decrease in internal oxygen, and an increase in internal carbon dioxide. A high but relatively constant ratio of gas concentrations between the central cavity of the fruit and the ambient atmosphere was observed for both ethylene and carbon dioxide, showing that a physiologically active ethylene concentration may exist in tissues, while the apparent rate of production is almost too low to detect. The role of ethylene remains obscure, but its concentration appears to be closely related to the ripening process in cantaloupes (*Cucumis melo* L. var. *reticulatis*, cultivar P.M.R. 45).

9:00. YOUNG, R. E., R. L. BIELESKI and J. B. BIALE, Univ. of California, Los Angeles 24, Calif. Phosphate esterification in tissue slices of the Fuerte avocado fruit.

Tissue slices from the preclimacteric (I) and from the climacteric peak (II) stages of the avocado fruit were incubated for short periods with labeled inorganic phosphate. The organic phosphates formed in the tissue were extracted, and separated by electrophoresis and by two dimensional chromatography. The labeled compounds were detected by autoradiography and their activities determined directly on the paper.

The rate of absorption of inorganic phosphate was much higher in stage (II) than (I). Dinitrophenol (DNP) did not alter this rate in (II) while it did in (I). Leakage of radioactive phosphates from the slices was greater in (II) than in (I) upon transfer to water or dilute phosphate.

P^{32} was rapidly incorporated into adenine, uridine, and guanosine nucleotides, hexose-phosphates, and into an as yet unidentified compound which in both solvents moved more rapidly than inorganic phosphate. The relative labeling in the total nucleotide fraction was much higher in (I) than in (II) while the reverse relationship was obtained with hexose-phosphates. P^{32} distribution in all nucleotides was over 40 per cent of total esterified in (I) and only 12 per cent in (II). DNP reduced the total organic labeling more markedly in (I) than in (II). Its effect was more striking on the nucleotide than on the hexose-phosphate fraction. The high activity in the unidentified compound in stage (II) for both the control and the DNP treatment has an important bearing on the evaluation of the uncoupling process in relation to the climacteric rise in respiration.

9:15. BEN-YEHOSHUA, S., R. M. ROBERTSON and J. B. BIALE, Univ. of California, Los Angeles 24, Calif. The respiration and the ripening of avocado fruit in relation to gaseous exchange and the internal atmosphere.

Mechanical stimulation or improvement of gaseous exchange is capable of accelerating the ripening process of avocado fruit by shortening the lag period that precedes the climacteric. By peeling the skin all or most of the lag period was eliminated and the onset of both the respiratory rise and ripening hastened. Removal of the skin during the postclimacteric stage resulted in a marked inhibition of the respiration rate. Mechanical handling produced similar effects.

A correlation between the composition of the internal atmosphere and respiration rate existed prior to fruit softening. During the respiratory rise oxygen percentage declined and the carbon dioxide rose. Following fruit softening the correlation changed. Despite the decline in respiratory activity the oxygen percentage decreased to values as low as 1 to 2 per cent and carbon dioxide percentage rose to 15%. This change in the correlation appeared to be associated with a rise of resistance to gaseous exchange. This rise in turn was related to the clogging of air spaces by cell exudates. Peeling the fruit resulted in a shift of the changes in internal atmosphere in accordance with expectations from the shift in the respiratory activity. The concentration of carbon dioxide was reduced slightly whereas the oxygen concentration was not affected significantly. Trials to demonstrate an autogenous volatile inhibitor other than CO_2 were unsuccessful.

It was concluded that the conditions in the internal atmosphere exerted a delaying effect on the onset of the climacteric rise. However, the delay was not ascribed to oxygen deficiency.

9:30. BEN-YEHOSHUA, S., G. G. LATIES and J. B. BIALE, Univ. of California, Los Angeles 24, Calif. The ripening process in discs of the avocado fruit.

The climacteric pattern of respiration was demonstrated in discs of avocado fruit kept in moist air. When water was used as the medium, the respiratory rise was inhibited, softening delayed, and senescence accelerated. These effects appeared to be unrelated to limitations in gaseous exchange. The respiratory activity of tissue slices was higher than that of intact fruit, but the difference was more pronounced in the pre- than in the post-climacteric stage.

A number of organic and inorganic substances used for tonicity studies prevented or delayed the ripening process. D-mannitol was the only osmoticum which accelerated softening as compared with water.

Metabolic inhibitors (DNP, Arsenite, Fluoroacetate) prevented the ripening process at low concentrations.

The leakage of cellular contents from discs immersed in water at room temperature increased with the development of the climacteric pattern. While leakage commenced prior to the onset of the rise, the rate climbed steeply along with the ripening process.

The apparent free space (AFS) of the avocado fruit was studied by the weighing method. The AFS of ripe fruit was much higher than that of freshly picked fruit.

The increase in AFS and in leakage was indicative of changes in the permeability of the cellular membranes along with the ripening process.

9:45. OBERBACHER, M. F. and H. M. VINES, Florida Citrus Commission, Lake Alfred, Fla. The use of gas chromatography for measurement of carbon dioxide and oxygen in small gas samples.

Gas chromatography was applied to the analysis of carbon dioxide and oxygen in small gas samples. A two column system with a set of detectors for each column was used. Two different sets of columns were investigated: (1) silica gel and molecular sieve and (2) hexamethyl-phosphoramide (HMPA) and molecular sieve. The first combination of columns retained the carbon dioxide on the silica gel column until oxygen and nitrogen emerged, then carbon dioxide was recorded as a broad peak. The latter combination gave a sharp carbon dioxide peak before the other gases were detected. The areas of the recorded peaks were correlated with the amount of the specific gas in the sample. Gas samples as small as 0.1 ml, depending upon the concentration, can be analyzed for these gases.

Internal gas contents of fruit were followed by removing a gas sample from within the fruit with a hypodermic needle and syringe and injected directly into the gas chromatography apparatus. Gas changes occurring within polyethylene bags, containing fruit, were followed by the same method. Analyses of internal carbon dioxide content of tangerines and carbon dioxide accumulation from fruit stored in polyethylene bags, will be discussed.

10:00. Recess.

10:15. TREGUNNA, E. B., G. KROTKOV and C. D. NELSON, Queen's Univ., Kingston, Ontario, Canada. Effect of light intensity during illumination on carbon dioxide evolution in the following dark period.

Detached tobacco leaves were placed into a closed-circuit apparatus and the air was continuously circulated over the leaves and through an infra-red CO_2 analyzer. Knowing the volume of the apparatus and the percentage of carbon dioxide in its air, the amounts of carbon dioxide either absorbed or evolved by a leaf were calculated.

When, after a period of illumination, leaves were darkened, the attainment of their steady rates of respiration was preceded by two outbursts of carbon dioxide evolution. Since these outbursts occurred only after a period of illumination, it has been concluded that both were the result of photostimulation. The peak of the first outburst was usually considerably higher than that of the second. This first outburst was of short duration and the height of its peak was accentuated by the increased light intensity in the preceding light period. The second outburst lasted longer and prior light intensity had no effect on the height of its peak.

10:30. HARLEY, J. L. and H. BEEVERS, Purdue Univ., Lafayette, Ind. Acetate utilization in maize roots.

A series of experiments with acetate-1-C^{14} and acetate-2-C^{14} has been performed with day old maize root tips. The object was to determine whether the appearance of C^{14} in the respired CO_2, the individual organic acids and the amino acids was fully consistent with the operation of the TCA cycle and known ancillary reactions. Further, it was hoped that estimates of the rate of cycling would be possible.

For at least 15 minutes organic acids and amino acids comprised the whole of the soluble labeled compounds. No C^{14} was found in sugars. The curve for per cent incorporation into organic acids extrapolated clearly to 100% at zero time and the incorporation into amino acids behaved in a reciprocal manner. Of the various acids of the TCA cycle, citrate was the first to become labeled. At 2 minutes it contained over 80% of the C^{14} in the acid fraction while succinate contained less than 10% and malate virtually none. In spite of its large pool size, malate was significantly labeled only after 4 minutes.

Parallel experiments on the progress of CO_2 emission at 1 minute intervals showed that no $C^{14}O_2$ was released from acetate-2-C^{14} until the 14th minute, that is, considerably later than the appearance of C^{14} in malate. By contrast, the release of C-1 of acetate as $C^{14}O_2$ began at about the same time as labeling was apparent in malate.

10:45. VARGAS, MIRIAM and C. O. CLAGETT, Pennsylvania State Univ., University Park, Pa. Fatty acids in germinating tobacco seed, effects of respiration.

Alcoholic extracts of germinating tobacco seed stimulate oxygen uptake in seedling homogenates and in mitochondria recovered from seed homogenates. The stimulation is limited to the mitochondrial fraction. The extracts were fractionated by paper chromatography followed by silica gel chromatography. The active principle chromatographed in the region of the high molecular weight fatty acids. Gas chromatographic fractionation indicated an enrichment of the linoleic acid and related acids in the most active cuts. Pure linoleic acid stimulates oxygen uptake to a small extent. Linolenic acid, 6 mol per Warburg flask, produces the same effect as the same molar concentration isolated from tobacco seed. Oxidative phosphorylation is uncoupled by linolenic acid and by the extract.

11:00. GRANT, B. R. and H. BEEVERS, Purdue Univ., Lafayette, Ind. Hexose absorption by carrot discs.

As part of a study of the mechanism of active uptake of non-ionic solutes by plant cells, the absorption of C^{14}-labeled glucose, fructose and galactose has been investigated. Rates of uptake from dilute solutions (0.001 M) have been determined at temperatures from 2°-30°. Washed carrot slices (in which the induced respiration has been allowed to develop) typically show a brief, rapid uptake of C^{14}-hexose, after which a linear uptake is maintained until the medium is depleted. The rate of this linear uptake is dependent on the temperature. Unwashed discs show a similar initial

absorption but this is followed by a lag of several hours before the linear phase of uptake develops. The relationship of changes occurring during the development of the induced respiration to the capacity for metabolic absorption of hexoses is being investigated.

11:15. REED, DONALD J., Montana State College, Bozeman, Mont. Glucose metabolism by etiolated sorghum seedlings.

The metabolism of glucoses labeled in the —1, —2, —3, 4, and —6 positions with C^{14} by etiolated *Sorghum vulgare* (var. Rox orange) seedlings has been investigated by the radiorespirometric method of Wang *et al.* (*J. Bacteriol.*, 76, 207 (1958). The results indicate that the germinating media plays a role in determining the relative contributions of the pathways of glucose metabolism.

Sorghum seedlings were grown in the dark for 3-4 days on stainless steel screens suspended over either aerated distilled water or an aerated 0.01 M inorganic nutrient solution of Mitchell *et al.*, (Agricultural Handbook No. 126, USDA 1958 p. 66). Respiration studies were conducted in either a 0.01 M KH_2PO_4 buffer (pH 4.9-5.0) or in the 0.01 M inorganic nutrient solution (pH 4.9-5.0). Seedlings grown in distilled water when placed in phosphate buffer for respiration measurements, show a very marked reduction in ability to metabolize glucose via the pentose phosphate pathway. In contrast, if the respiration studies are conducted in the inorganic nutrient solution after distilled water germination, a much greater portion of the glucose is metabolized via the pentose phosphate pathway. In addition the respiration rate of seedlings grown in the inorganic nutrient solution, does not appear to be affected appreciably by phosphate buffer. The presence of 10^{-3} M 2,4-D in the respiration media markedly increases the pentose phosphate pathway over the glycolytic pathway.

11:30. HANSON, J. B., Univ. of Illinois, Urbana, Ill. Studies on the uncoupling action of ribonuclease applied to corn shoot mitochondria.

Preincubation of mitochondria with pancreatic ribonuclease will uncouple phosphorylation and impair oxidation (Hanson, *J. Biol. Chem.* 234:1303, 1959). Comparable studies with protamine and polylysine suggest that part of this effect is due to binding of a basic protein to negatively charged phosphorylation sites. It appears that most di- or polyvalent cations except Mg have an uncoupling effect. Even Mg is inhibitory in high concentration.

However, RNase appears to have an uncoupling action over and above that due to its properties as a basic protein. In low concentrations it will sometimes increase substrate oxidation rates, a property not shared by other basic proteins. The hydrolysis of RNA considerably reduces the negative sites capable of binding Ca^{45}. The presence of Mg during preincubation will partially protect the mitochondria from the action of RNase, and there is a correlation between RNA remaining and phosphorylation rates.

This type of uncoupling seems to be somewhat different from that produced by DNP. DNP, for instance, potentiates an accelerated electron transport in a system limited by Mg; this is not true for RNase. A postulate for two types of uncoupling has been formulated; one type involves cationic inhibition of phosphorylating sites, the other a shunting of electrons around these sites.

11:45. KIDDER, GEORGE W., III, Univ. of Pennsylvania, Philadelphia, Pa. Studies on the inhibitor resistant respiration of *Myrothecium verrucaria.*

The respiration of the intact mycelium of *Myrothecium verrucaria* was shown by Darby and Goddard in 1950 to be insensitive to cyanide and carbon monoxide, although the cytochrome oxidase activity of crude extracts was sensitive. Further experiments with this organism are here reported.

The whole mycelium is insensitive to 2×10^{-4}M NaN_3 and 50 percent inhibited at about 5×10^{-2}M, although these values fluctuate with culture age. Carbon monoxide inhibits only at very high CO/O_2 ratios, while cyanide inhibition roughly parallels that of azide.

A particulate fraction of homogenized mycelium has been isolated under conditions appropriate to plant mitochondria. Spectroscopic observations at low temperature show cytochrome pigments tentatively identified as *c*, an *"a"* type pigment, and two *"b"* type pigment. These last were oxidized in the presence of azide, and reduced in the presence of inhibitory concentrations of antimycen A. These spectra show similarities both to higher plant and animal cytochrome systems.

These particulate preparations can oxidize succinate, α-ketoglutarate, and citrate with stimulation of these rates by ADP. DPNH is rapidly oxidized, and the preparation shows a high cytochrome oxidase activity which is inhibited by cyanide, azide and carbon monoxide to about ¼ of the uninhibited rate by low concentrations of the inhibitors. Further inhibition requires very high inhibitor concentrations. This effect may account in part for the inhibitor insensitivity of the organism.

12:00. BAKER, JAMES E., Plant Industry Station, Beltsville, Md. Effects of diphenylamine on electron transport in plant mitochondria.

Succinoxidase activity of sweet potato mitochondria is inhibited 50% by 3.0×10^{-4} M diphenylamine (DPA). Spectrophotometric observations indicated that cytochromes of the mitochondria were reduced in the presence of DPA but at a slower rate. Turnip mitochondrial succinoxidase was inhibited 90% by the same concentration of DPA. In the latter system, no reduction of cytochromes occurred during a time interval in which almost complete reduction of cytochromes occurred in controls. DPNH oxidase, DPNH-cytochrome *c* reductase, and succinate-cytochrome *c* reductase activities of both sweet potato and turnip mitochondria were inhibited by DPA. These results place the site of DPA inhibition somewhere between substrate and the cytochrome chain, probably at the flavorprotein level.

Under conditions of these experiments, the sweet potato system is inhibited approximately 50% by cyanide and antimycin A. Antimycin A completely inhibited the residual 50% of the succinoxidase activity in the DPA-inhibited system. If an alternate pathway exists, this result means that the ability of the particles to oxidize succinate via this pathway has been blocked by DPA. The DPA effect is not con-

fined to cyanide- and antimycin A- resistant electron transport systems, however, since the turnip system is completely inhibited by antimycin A and azide. Depending on the specificity of DPA action, a new approach to the problem of cyanide-resistant respiration may be open. A differential effect of DPA on oxidative phosphorylation will be discussed in relation to similar effects produced by other respiratory-chain inhibitors.

12:15. STOTZKY, GUENTHER, WALTER CULBRETH and LAWRENCE B. MISH, United Fruit Co., Norwood, Mass. An apparatus for growing intact plants with aseptic roots.*

An apparatus has been developed for growing plants with aseptic roots for studies on root respiration and the biochemical nature of root exudates. The apparatus consists of 1) a growth tube into which a surface-sterilized seed or sterile seedling is planted, 2) a root chamber containing a solid growth medium, 3) an irrigation chamber, and 4) a nutrient solution reservoir. The seed and above-ground portion of the plants are separated spatially from the root zone, in order that products from the endosperm do not contaminate the root exudates. The photosynthetic and respiratory activities of the above-ground portions, which are allowed to develop in air, are separated from the respiration of the roots by a sealing material poured around the plant stem. A continuous CO_2-free air stream, sterilized through in-line filters and distributed to individual culture units through a manifold, aerates the roots and removes respired CO_2 for analysis. The roots are periodically perfused with a nutrient solution, which also washes the root exudates into the irrigation chamber. The exudates are removed for analysis under mercury, to prevent microbial contamination. Provisions are made for aseptic sampling of the root-zone atmosphere for gas chromatographic analysis and for the introduction of microorganisms or other materials. The apparatus is compact and is autoclaved as a unit. Mono- and dicotyledonous plants have been grown successfully in these units. Representative exudate and respiratory patterns will be presented.

SESSION E4. Room 4, Memorial Center

TRANSLOCATION AND ION UPTAKE

E. Epstein, *presiding*

8:25. Announcements.

8:30. GALLOWAY, R. A. and R. W. KRAUSS, Univ. of Maryland, College Park, Md. Utilization of phosphate compounds by *Chlorella*.

Pyrophosphates are key compounds in the phosphate economy of cell metabolism, and are also currently assumed to play a role in the Emerson Effect. Tetra sodium pyrophosphate was tried as a source of phosphorous for *Chlorella pyrenoidosa*. Neither phosphate-sufficient nor phosphate-starved cells, in cultures at any pH from 4.5 to 9.0 in the light or dark, were able to use pyrophosphate. A reported increase of photosynthetic enhancement by pyrophosphate appears, therefore, to be some secondary phenomena not directly associated with photosynthesis. This lack of utilization can be attributed to a failure to absorb pyrophosphate. Analyses confirm that no pyrophosphate was absorbed from the medium. The ability of Chlorella to utilize other phosphate compounds will be discussed.

8:45. SANSING, N. G. and C. W. WHITEHEAD, Iowa State Univ., Ames, Iowa. Translocation of P^{32}.

P^{32} applied to intact leaves has been translocated into various tissues of soy bean, tomato and beet plants, with 2 to 10% of the applied P^{32} moving out of the leaf. Translocation from the leaf has increased with age to approaching senility. Downward movement in stem or petiole has been stopped by killing the tissues with a hot paraffin ring; upward movement was not affected.

Cooling tomato stems with a water coil has reduced downward translocation of P^{32} to near zero. With rising stem temperatures, downward translocation has increased linearly.

Ringing stems, above or below the point of P^{32} application; chilling the stem, or pinching out the apical bud of the plants has reduced translocation from the treated leaf. Chilling the lower stem of tomatoes has reduced total translocation from the treated leaf by nearly 70%, most of the reduction being in phosphorous that would normally have been moved upward.

9:00. SCHOPMEYER, C. S., Beltsville, Md. Absorption and translocation of foliarly applied phosphorus by loblolly pine seedlings.

Loblolly pine seedlings were grown for this study in water cultures in a greenhouse. Phosphorus content of the needles at the time of treatment was 1.3 mg per gm of dry tissue, a level just above minimum sufficiency.

Treatment consisted of briefly immersing the foliage and stem of a seedling in 0.5 M. $(NH_4)_2\ HP^*O_4$ containing 50μc of P^{32} per gm of total P and 500 ppm of the wetting agent—"Tween 20." Treatment resulted in the deposition in or on the needles of 5.6 mg of P* per gm of dry tissue.

The amount of P* translocated to the root tips was appreciable 24 hours after treatment and was near maximum in samples collected 48 hours after treatment.

Of the total amount of P* deposited on the foliage and stem, 8.2 percent was recovered, 3 weeks after treatment, from the untreated portions (roots and new shoots).

Needles from new shoots which developed during the 5 weeks following treatment contained 2.3 mg of total P per gm of dry tissue. Of this amount, 65 percent was P* which had been translocated from the foliar deposit.

Height growth of the treated seedlings was reduced to a rate only 60 percent of that for seedlings having 50 ppm of P available in culture solutions to the roots. A test revealed that this reduction in height growth was not caused by the radioactivity of the tracer. The possible effect of "Tween 20" on height growth was not checked.

9:15. SHIROYA, MICHI, G. R. LISTER, C. D. NELSON and G. KROTKOV, Queen's Univ., Kingston, Ontario, Canada. Translocation of C^{14} in tobacco at different stages of development following assimilation of $C^{14}O_2$ by a single leaf.

Single leaves of tobacco plants at four different stages of development were allowed to carry on photosynthesis in $C^{14}O_2$ for 30 to 50 minutes followed by photosynthesis in air. The total elapsed time from the beginning of assimilation was 5½ hours. Redistribution of C^{14} was determined in I a young plant with 8 leaves, II an older plant with 19 leaves, III a mature flowering plant with 29 leaves and IV a mature seed plant with 35 leaves. The leaf treated with $C^{14}O_2$ in each of the four plants was number seven, counting from the bottom. This leaf was only half expanded in the youngest plant but fully expanded in the other three plants.

The treated leaf of the youngest plant translocated 22% of the total C^{14} fixed, twice as much as the treated leaves of the other three plants. The large amount of translocation in the youngest plant occurred from a leaf that contained 28% of its ethanol-soluble fraction as sugar phosphates and only 25% as sucrose. The treated leaves of the older plants that translocated half as much radioactivity had less C^{14} in sugar phosphates and more C^{14} in sucrose. For example the treated leaf of the seed plant contained 13% of its soluble C^{14} in sugar phosphates and 70% in sucrose.

Sucrose and some hexose was recovered from the stems and roots of all plants. Amino acids were recovered from both stem and root of the youngest plant but not from these parts of the three older plants. Since 80 to 100% of the C^{14} in the stems was recovered as sucrose it is concluded that this sugar is the main form in which carbon is translocated in tobacco. Although sugar phosphates and organic acids were found in the treated leaves of all plants they were not detected in stems or roots and were apparently not translocated.

All plants translocated C^{14} to leaves both below and above the treated leaf. Two factors influenced the destination of C^{14}. One was the anatomical connection among leaves and the second was the stage of development of any particular leaf. Although C^{14} was translocated to leaves at nodes bearing flowers no C^{14} was translocated to these flowers or to developing or mature seeds in any of these plants.

9:30. MORTIMER, D. C., N.R.C., Ottawa, Canada. Translocation of photosynthetic products in sugar beet petioles.

The translocation of radioactive sucrose, formed during and after the photosynthetic assimilation of $C^{14}O_2$ in the sugar beet leaf, has been investigated under a variety of conditions. The negative gradient of radioactive sucrose in consecutive, small pieces of petiole (from leaf to base) obeys a linear rather than a logarithmic function. A maximum level of labelled sucrose is attained at the top of the petiole 30 minutes after the one minute period of assimilation. After an additional 30 minutes the whole petiole has "filled" to the same level. There is no indication of metabolism of the radioactive sucrose in the petiole for at least two hours. The apparent velocity of transport is not changed by chilling the petiole to 10° or 20° C or warming it to 33° C. Chilling a small section of the petiole to 2° C blocks movement past the chilled region. Hydrogen cyanide administered to the petiole and local anaerobic conditions do not affect the apparent velocity. It is, however, relatively easy to control the amount of radioactive sucrose entering the conducting tissue from the leaf by correctly timed darkening, chilling, warming, or cyanide inhibition of the leaf blade. The concentration of radioactive carbon dioxide administered during the initial one minute influences the quantity and apparent velocity of sucrose translocation.

9:45. MITCHELL, JOHN W. and PAUL J. LINDER, U.S. Dept. of Agriculture, Beltsville, Md. Effect of alpha-methoxylation and nitrogen acetylation on absorption and translocation of a plant regulator, methyl indole-3-acetate.

Alpha-methoxylation of several plant-growth-regulating chemicals enhanced their absorption and translocation by plants. Alpha-methoxylation has now been extended to methyl-indole-3-acetate, a growth regulator of the indole type, with the result that the absorption and translocation of this regulator were also enhanced. Alpha-methoxylation together with nitrogen acetylation, however, resulted in greater translocatability of the methyl indole-3-acetate entity than did the methoxylation alone.

10:00. Recess.

10:15. MITCHELL, JOHN W., PAUL J. LINDER and MELBA B. ROBINSON, U.S. Dept. of Agriculture, Beltsville, Md. Root exudation of alpha-methoxyphenylacetic acid.

Results of experiments designed to obtain a better understanding of root exudation and the mechanism involved following application of C^{14}-labeled alpha-methoxyphenylacetic acid (MOPA) to plants were studied. This plant regulator was absorbed by the leaves of bean plants, translocated to the roots, and exuded into aerated water without detectable chemical change. The compound was present in roots of plants following its application to the leaves. No C^{14} metabolites of this compound were detected in the roots or in water surrounding them.

Exudation of MOPA did not reach a maximum rate until the amount in the roots reached a maximum. The amount of MOPA exuded by the roots was proportional to the amount of the regulator applyied to their leaves over a dosage range up to 40 times that required to bring about a barely detectable amount of exudation. Exudation of MOPA from roots was proportional to the amount of intact root remaining attached to the above-ground parts of the plant. Comparison of rates of exudation showed that 20% of MOPA applied to the leaves exuded from the roots and that only 3% of C^{14}-labeled 2,4-D applied to the leaves moved out of the roots.

10:30. TANADA, T., U.S. Dept. of Agriculture, Beltsville, Md. Localization of calcium enhancement of rubidium absorption in the mung bean root.

The largest effect of calcium on increasing Rb uptake by mung bean root segments was found to be in the 2.5-5.0 mm segment from the tip. Calcium was less effective in increasing Rb uptake near the tip and in the mature regions. Calcium was necessary during the growth of the roots in order for it to bring about large increases in Rb absorption.

10:45. EPSTEIN, E., Univ. of California, Davis, Calif. Essentiality of calcium in selective ion transport.

Mutual effects of monovalent ions on each other's absorption by barley roots were studied in short-term experiments of up to about one hours's duration. Both in the absence and presence of Ca^{++}, K^+ and Rb^+ compete with each other, indicating identical carrier sites for these two ions. Absorption of K^+-Rb^+ from 1mM solutions is scarcely affected by even a 20-fold excess of Na^+ ions, provided that Ca^{++} is present at about 0.05-0.1 mM concentration. Though without effect on K^+-Rb^+ absorption under these conditions, Na^+ is nevertheless absorbed. This demonstrates the existence of Na^+ carrier sites distinct from those that carry K^+-Rb^+.

In the presence of Ca^{++}, absorption of Na^+ is inhibited at low concentrations of K^+, but at progressively higher K^+ concentrations there is little or no further interference with Na^+ absorption by K^+. This again bears out the conclusion that there are Na^+ transporting sites distinct from those that carry K^+-Rb^+.

In the absence of Ca^{++} the rate of K^+-Rb^+ absorption declines in the presence of Na^+ and falls to nil in about one hour. Other divalent cations besides Ca^{++}, notably Mn^{++} and Zn^{++}, maintain the integrity of the selective absorption mechanisms, though less effectively than Ca^{++}. The Mg^{++} ion is largely ineffective.

These and related experiments will be presented and the requirement for Ca^{++} in selectivity will be discussed.

11:00. WAISEL, YOAV, Univ. of Missouri, Columbia, Mo. The absorption of lithium and calcium by barley roots.

The competitive inhibition of Lithium uptake by Calcium was demonstrated under various experimental conditions and pretreatments. Strontium, known to inhibit Ca absorption competitively also competes with Li. Magnesium does not show a competitive inhibition either of Ca or of Li absorption.

The Q_{10} of the Li absorption process (20° C-30° C), both from a pure solution and from a Li + Ca solution, is about 2.3-2.5 This points to a chemical reaction and excludes the explanation, given in literature, of the formation of a barrier by calcium.

Competition on sites is seen also at 0° C where no metabolic uptake is presumed to take place. This might indicate that at least a portion of the cations is adsorbed on the carriers. As revealed by exchange trials the specificity of some of the sites for Li is rather high.

The relationship between the non-metabolic and the metabolic phases, was studied by immersing roots in Li solutions at 0° C followed by an interval in water at 30° C or at 0° C and a return back to a Li solution at 0° C. The "absorption shoulders" seem to be formed by reloading at O° C of the sites which were unloaded during the interval at 30° C by the metabolic uptake. No "shoulder" was formed following an interval in water at 0° C where no metabolic uptake could take place. "Shoulder" formation could be successively repeated and no decrease in its ability to be formed was found even after a prolonged preincubation in water at 0° C. Thus, another explanation is given to the "shoulder" formation beside the generation of Patent-ion-transport capacity.

11:15. LEGGETT, J. E. and L. H. STOLZY, USDA, Beltsville, Md. and Univ. of California, Riverside, Calif. Uptake of sodium by barley plants.

Barley plants accumulated sodium under both aerobic and anaerobic conditions. Steady state uptake by the roots was attained after one minute in an aerobic system, in comparison to 30 minutes anaerobically. In either condition, sodium accumulation by the shoots was linear with time after 45 minutes. The rate of accumulation by both roots and shoots was decreased under anaerobiosis. The inhibition produced by anoxia was reversible upon addition of oxygen; in fact, the amount of sodium appearing in the shoots was increased. This activation of uptake by the shoots was also inhibited by the absence of oxygen.

Inhibition produced by nitrogen and carbon monoxide was the same order of magnitude. Addition of oxygen for short periods of time reversed the inhibition in roots by CO and in shoots by N_2, without reversal of inhibition of the respective shoots and roots. Long periods of aeration would reverse the inhibition produced in the presence of CO and N_2. The inhibition of accumulation by shoots in the presence of CO can be reversed in short times by treatment of plants with light during aeration.

Lithium concentrations $< 10^{-3}$ M were without effect on sodium uptake by roots and shoots. Both K and Ca inhibited uptake by the roots. Accumulation by shoots was decreased slightly by K and increased by low Ca concentrations.

The relationship of sodium accumulation by roots and shoots in the presence and absence of oxygen and other inhibitors in absorption solutions will be discussed.

11:30. TANADA, T., USDA, Beltsville, Md. Non-steady state kinetics of rubidium absorption by barley roots.

The absorption of Rb by excised barley roots was studied under non-steady state conditions to determine whether at low Rb concentrations the reaction resolves into a first-order reaction. The reaction was found not to conform to first-order kinetics down to 10^{-6} M Rb concentration.

11:45. HIGINBOTHAM, N., BUD ETHERTON and R. J. FOSTER, Washington State Univ., Pullman, Wash. The source and significance of the electropotential of higher plant cells.

Theoretical considerations require that both the concentration and the electropotential gradients be known in order to establish that an ion is actively (energetically) absorbed by a cell. Thus measurements of the electropotential difference (PD) between cell interior and exterior may afford the means of distinguishing which ions are metabolically transported through the cell membrane. Data on the influence of various external solutions has been obtained which shows that external Ca plays an important role in maintenance of the membrane PD. The anion accompanying Ca has little effect over the concentration range studied. The evidence thus far obtained suggests the existence of an energetic cation efflux process rather than one for anions.

12:00. ETHERTON, B., Washington State Univ., Pullman, Wash. The use of microelectrode measurements of cellular potentials in the study of active transport in plants.

Experiments were performed to test for the occurrence and to ascertain the direction of active transport mechanisms for sodium and potassium ions in the cells of oat and pea seedling tissues.

The criteria used for active transport are based on the equation

$$\frac{\text{flux in}}{\text{flux out}} = \frac{(M^+)\text{ out}}{(M^+)\text{ in}} \quad \exp.\ z\,(.039)E \qquad (1)$$

where (M) is the concentration of any cation species, z is its charge, and E is the transmembrane potential in millivolts. If the ion species under consideration is in equilibrium, the above equation simplifies to

$$(M^+)\text{ in} = (M^+)\text{ out}\exp.\ z(.039)E \qquad (2)$$

Both of the above equations describe how an ion will be distributed if it moves passively according to electrical and concentration forces. For example, if the transmembrane potential were 120 mV. and the external concentration of a cation were 1 mM., then the calculated internal concentration of a passively distributed ion would be 100 mM. If the internal concentration were higher, then active uptake of the ion would be suggested, and vice versa.

In accordance with the above considerations, the transmembrane potentials of individual cells were measured with microelectrodes when the tissues were in equilibrium with sodium and potassium ions. Also, the internal and external concentrations of sodium and potassium ions were measured.

The experimental results suggest that sodium ions were actively extruded at all external concentrations used. In the case of potassium, the evidence suggested active accumulation at low and active extrusion at high external concentrations.

WEDNESDAY AFTERNOON
CONCURRENT SESSIONS F1, F2, F3, F4

SESSION F1. Room 310, Memorial Center

Informal Discussion: **ENERGY TRANSFER IN PHOTOSYNTHESIS**

R. K. Clayton, *presiding*

1:30.

SESSION F2. Room 318, Memorial Center

BIOCHEMISTRY III

H. W. Siegelman, *presiding*

1:30. PALLAS, JAMES E., Jr., Southern Piedmont Soil Conservation Field Station, Watkinsville, Ga. Starch accumulation in guard cells.

The guard cells of dark-grown seedlings of *Phaseolus vulgaris L., Zea mays L.,* and the destarched leaves of many other species accumulated starch in high concentrations from externally supplied sugars, especially sucrose. The sugars were supplied by cutting the tip of a leaf and immersing the cut edge, or by floating leaf pieces and epidermal strips in solutions under controlled conditions.

Mesophyll and leaf bundle cells were active in the transformation and translocation of sugars to the guard cells. Evidence of this will be presented.

Destarching of the guard cells was found to be difficult in some species, requiring prolonged periods of semidarkness. Some starch usually remained in the guard cells after death of an individual leaf during the destarching period.

True albino corn plants grown on .3M sucrose also showed the ability to accumulate starch in their guard cells, and their stomata opened, indicating that the guard cells were operative.

The implications of the results with respect to guard cell operation will be discussed.

1:45. PUNNETT, THOMAS and ETHYL C. GREIG, Univ. of Rochester, Rochester, N.Y. The chemical composition of algal cell walls.

Cell walls of five species of unicellular algae from the *Cyanophyta* and *Chlorophyta* have been prepared by grinding and repeated washing. The amino acid composition of the walls has been determined by hydrolysis and two-dimensional chromatography analysis. In all species, eight different amino acids are found, namely glutamic acid, aspartic acid, alanine, glycine, serine, valine, leucine and isoleucine. In addition to these, the wall of each species possesses from one to four additional ninhydrin-positive compounds which are characteristic of the species. These results as well as the carbohydrate, lipid and pigment analyses will be discussed.

2:00. RENDIG, V. V. and E. A. McComb, Univ. of California, Davis, Calif. Some anomalous effects on carbohydrate composition of plants "fed" L-sorbose.

The introduction of L-sorbose into intact plants results in the formation and accumulation, in the leaves, of carbohydrates which are not normally present, at least in amounts great enough to be detected chromatographically. This effect of L-sorbose is unique when compared with eighteen other carbohydrates, including a tetrose, pentoses and hexoses having both the D- and L- configuration, and sugar alcohols.

By means of radioautography and paper chromatography the accumulation of four unexpected carbohydrates was revealed in L-sorbose "fed" plants. One of these compounds has been tentatively identified as L-galactoheptulose. As far as we are aware this heptulose has not been reported previously to be present in higher plants but has been produced from the naturally occurring sugar alcohol, perseitol (D-glycero-D-galacto-heptitol) by the action of *Acetobacter suboxydans.*

The necessary physiological conditions for producing these anomalous carbohydrates and the effects of other sugars, metabolic inhibitors, etc. will be discussed.

2:15. LOEWUS, F. A. and S. KELLY, U.S. Dept. of Agriculture, Albany, Calif. Uronic acid metabolism and ascorbic acid synthesis in the detached strawberry.

The metabolism of D-galacturonic acid-1-C^{14} and the methyl ester of D-galacturonic acid-U-C^{14} have been studied in the detached ripening strawberry. The principal products of D-galacturonic acid-1-C^{14} to become labeled are L-ascorbic acid-6-C^{14}, L-galactonic acid-6-C^{14}, galactaric acid-C^{14} (COOH labeled), pectin-derived D-galacturonic acid-1-C^{14} and D-xylose-1-C^{14} among the free sugars. The methyl ester contributes label primarily to L-ascorbic acid and pectin-derived D-galacturonic acid, both uniformly labeled. Comparison will be made between these results and previous experiments involving the metabolism of D-glucuronolactone-1-C^{14} and -6-C^{14} in the same plant (*Biochim. Biophys. Acta* 30 (1958) 629; 38 (1960) 332).

2:30. BARBER, G. A., Univ. of California, Berkeley 4, Calif. *In vivo* oxidation of L-rhamnose and L-glucose by higher plants.

In the course of a study of rhamnose metabolism in plants L-rhamnose was administered to green buckwheat seedlings (*Fagopyrum esculentum*) through their excised stems. Upon paper electrophoresis, an extract of the seedlings was found to contain a compound subsequently shown to be rhamnonic acid. In addition to L-rhamnose a number of other monosaccharides were administered to the seedlings, but extensive accumulation of an aldonic acid occurred only in those exposed to L-glucose.

For characterization, the aldonic acids were isolated by elution from columns of Dowex-1 (formate). An aliquot of each acid was mixed with chemically prepared L-rhamnonic-1-C^{14} or D-gluconic-U-C^{14} acid and purified by paper electrophoresis. The radioactive acids were eluted, lactonized, and reduced to aldoses with $NaBH_4$ at O°. Lactones and aldoses prepared from the plant acids coincided with the radioactive standards upon co-chromatography. Evidence for the L configuration of the gluconic acid was provided by the optical rotation of its lactone and by the failure of its reduction product, presumably L-glucose, to act as substrate for D-glucose oxidase.

Green seedlings of cotton, barley, mung beans, and peas were tested, and all were found able to oxidize L-glucose and L-rhamnose to the aldonic acids.

2:45. PRICE, C. A., Rutgers—The State University, New Brunswick, N.J. D-lactate dehydrogenases in *Euglena gracilis*.

The rates of oxidation of D- and L-lactates by intact *Euglena gracilis* (Klebs), Z strain, are closely dependent on the zinc supply of the cells; i.e., $-q_{O_2}$ (PN) of zinc deficient cells are 5-10% of zinc sufficient controls. The total lactate dehydrogenase (LDH) activities of cell homogenates are also dependent on the zinc level, but those of zinc-deficient homogenates are higher than expected (25% of controls).

The finding of zinc-*independent*, pyridine nucleotide-dependent D-LDH activities indicated the existence of two or more LDH components, one of which must be particularly sensitive to zinc.

Intracellular fractionations of *Euglena* homogenates revealed essentially no activity in the cell wall-paramylon granule fraction and substantial activities in the mitochondrial and "soluble" fractions. The mitochondrial LDH was pyridine-nucleotide independent.

The hypothesis that mitochondrial LDH is the enzyme responsible for the zinc requirement for lactate oxidation by intact *Euglena* will be examined.

3:00. Recess.

3:15. SHANNON, L. M. and ABRAHAM MARCUS, U.S. Dept. of Agriculture, Beltsville, Md. An aldol cleavage of γ-hydroxy γ-methyl α-ketoglutarate to pyruvate.

γ-hydroxy γ-methyl α-ketoglutarate (parapyruvate) is a potent inhibitor of α-ketoglutaric oxidase [*J. Biol. Chem.* 221, 359 (1956)]. Its presence in plant tissue suggests that metabolic systems may be available for its removal. This report describes the isolation of an enzyme from cotyledons of germinating peanuts catalyzing an aldol type cleavage of parapyruvate to pyruvate. The enzyme has been purified 100 fold by acid precipitation and fractionation with acetone and $(NH_4)_2\ SO_4$. The purified enzyme shows an absolute requirement for a sulfhydryl compound, as well as a strong Mg^{++} requirement. The optimum pH for enzymatic activity is approximately 9.0. The affinity of the enzyme for parapyruvate is quite high ($Km = 6x10^{-5}M$).

In experiments designed to ascertain if the reaction were reversible, it was observed that the enzyme catalyzed a rapid exchange of C^{14}pyruvate into parapyruvate. The exchange reaction showed the same requirements as the cleavage reaction. The radioactivity product formed by the exchange reaction was identified by its 2,4 dinitrophenylhydrazone and by its transamination to γ-hydroxy-γ-methyl glutamate by a crude pigeon-liver preparation. Degradation of radioactive product indicated the exchange reaction was asymmetric involving only one pyruvate moiety. The implications of these findings on the mechanism of the reaction will be discussed.

3:30. HIATT, A. J., Univ. of Kentucky, Lexington, Ky. Condensing enzyme from tissues of higher plants.

Enzyme extracts containing condensing enzyme activity were prepared from acetone powders of plant mitochondria. The enzyme was present in every plant tissue in which it was sought, These sources included both leaves and roots of tobacco, soybeans, spinach, beans, and peas. The extracts were prepared by stirring acetone powders of plant mitochondria with 0.02 M potassium phosphate buffer, pH 7.4. The enzyme remained in the supernatant solution after centrifugation at 30,000 x G for periods up to two hours. Condensing enzyme was assayed by measuring citrate synthesis or by coupling to malic dehydrogenase and measuring DPN reduction. When assayed by measuring citrate synthesis, activity was absolutely dependent upon the presence of both acetyl CoA and oxalacetate.

The enzyme was purified 10-fold by absorption upon calcium phosphate gel and by ammonium sulfate fractionation. The partially purified enzyme was saturated by concentrations of 10^{-4} M acetyl CoA and 4×10^{-4} M oxalacetate. The K_M for acetyl CoA was 1.8×10^{-5} M. The enzyme

exhibited a broad pH optimum with maximum activity at approximately pH 8.4. Partially purified condensing enzyme was very stable and could be stored for several weeks at 2-4° C without loss of activity.

3:45. CRAFT, C. C., U.S. Dept. of Agriculture, Beltsville, Md. Localization and activity of phenolase in the potato tuber.

Phenolase activity was present in the particulate and soluble fractions of homogenates prepared from potato tissue. The relative activity of the fractions as well as the catecholase/cresolase ratios depended on the techniques of isolation. The particulate fractions with bound phenolase showed good rates of oxygen uptake when provided with α-ketoglutarate or succinate substrates. The copper enzyme inhibitor, phenylthiourea, at 5×10^{-3}M, almost completely inhibited phenolase activity, without affecting respiration of freshly-cut potato discs. However, the respiratory rise that typically occurs in potato slices following overnight aeration was partially inhibited by 5×10^{-3}M phenylthiourea. Similarly, the accumulation of chlorogenic acid and the formation of lignified barrier layers were retarded in the slices aerated in phenylthiourea, but the degree of retardation was not in proportion to the almost complete inhibition of phenolase activity. Phenylthiourea cannot be regarded as a specific inhibitor of phenolase activity in potato slices, since symptoms of general toxicity, as measured by ion leakage, were evident in the treated slices.

4:00. BUCKLEY, E. H., United Fruit Co., Norwood, Mass. Further studies on the biosynthesis of 3-hydroxytyramine in the peel of the banana.

3-Hydroxytyramine synthesis in the peel of the ripening banana fruit was studied through the use of C^{14}-labelled phenylalanine, tyrosine, DOPA (3,4-dihydroxyphenylalanine) and tyramine (Buckley & Towers, Abstr., Can. Soc. Plant Physiol. Meetings, May 24-26, 1961). These studies suggested that the pathway for 3-hydroxytyramine synthesis in the peel is tyrosine ⟶ tyramine ⟶ 3-hydroxytyramine.

Further work has revealed that only those peel tissues which accumulate 3-hydroxytyramine during ripening are capable of converting C^{14}-tyrosine to 3-hydroxytyramine. Since all peel tissues readily convert tyramine to 3-hydroxytyramine the synthesis appears to be blocked at the tyrosine ⟶ tyramine step. This hypothesis received some substantiation when the addition of pyridoxal phosphate increased the conversion of C^{14}-tyrosine to 3-hydroxytyramine, presumably by acting as a co-factor for tyrosine decarboxylase.

The reason for the changing pattern of 3-hydroxytyramine synthesis is unknown, although it does appear to be related to seasonal variations in the fruit.

4:15. PALMER, J. K., United Fruit Co., Norwood, Mass. The polyphenoloxidase of banana fruit.

The browning reactions of the banana fruit result primarily from the oxidation of 3-hydroxytyramine (DOPamine; 3,4-dihydroxyphenethylamine) to insoluble pigments. This oxidation is catalyzed by polyphenol oxidase, and some properties of the banana enzyme have been determined.

The polyphenoloxidase of banana fruit pulp is soluble in 1% detergent in 0.1 M potassium phosphate, pH 7.0. It catalyzes the oxidation of 3-hydroxytyramine via various quinones to insoluble brown pigments. Under appropriate conditions, the first stable product of the reaction is a colored quinone, 2,3-dihydroindole-5,6 quinone, and the activity is conveniently determined by measuring the rate of increase in optical density at 475 mμ when enzyme and substrate are mixed. Oxygen uptake has also been used to measure activity.

This relatively crude enzyme is not specific for 3-hydroxytyramine, but also catalyzes the oxidation of other ortho diphenols (catechol, chlorogenic acid, DOPA, adrenaline, DL-arterenol) at 60-100% the rate for 3-hydroxytyramine. Ascorbic acid, tyrosine and tyramine are not oxidized. The pH optimum for the oxidation of 3-hydroxytyramine is about 7.0, and the preparation retains its activity for weeks at 3° C.

The enzyme exhibits the product inactivation typical of a polyphenoloxidase and is also inhibited by reducing agents and by various reagents which complex copper.

Purification studies are continunig and additional properties of the purified enzyme(s) will be reported.

SESSION F3. Room 4, Memorial Center

GROWTH REGULATORS III

W. A. Andreae, *presiding*

1:30. LOCKHART, J. A., Univ. of Hawaii, Honolulu 14, Hawaii. Physiological mechanism of action of stem growth inhibitors.*

A number of chemical compounds which retard plant stem elongation have received increasing attention in the last few years, especially with respect to practical applications. These materials, exemplified by "Phosfon-D" and chlorocholine chloride have rather spectacular influences on stem elongation, while permitting essentially normal plant development. It would be of considerable theoretical interest to discover what hormonal or growth system in the plant is influenced by these compounds.

It has been found that Phosfon-D and gibberellin A_3 show a complete interaction on Pinto beans. This indicates that Phosfon-D is acting by decreasing the natural gibberellin supply to the elongating regions of stem and petioles. Further experiments indicate that Phosfon-D acts by moving to the plant stem even though treatment is made to the soil. It is only necessary to treat a portion of the roots to achieve a full inhibitory effect. The fact that these growth retarders act through one hormonal system makes them extremely useful for studies on the hormonal interrelations in stem growth.

1:45. CATHEY, H. M., Plant Industry Station, Beltsville, Md. The relation of phosfon structure to its growth-retarding activity.

Various chemical modifications of phosfon (tributyl-2,4-dichlorobenzylphosphonium chloride) were tested on the growth of snapbean, chrysanthemum, corn, cucumber, Easter lily, and poinsettia. A substitution of Cl, Br, or a methyl group in the para position on the benzene ring was essential for growth-retarding activity on bean and chrysanthemum grown

in treated soil. A substitution of a NO_2 group in the para position resulted in a compound as inactive as the unsubstituted benzyltributylphosphonium chloride. Substitutions of Cl, Br, or a methyl group in the ortho and meta positions on the benzene ring did not alter the activity of compounds with the para position occupied by the same groups, while those with the para position unoccupied were relatively inactive. All substitutions for the benzyl group including straight chains were inactive. Substitutions of chains shorter than four carbons around phosphorus made the compounds inactive. All the chemicals were toxic when applied to the foliage. Easter lily responded only to phosfon and closely related structures, while corn, cucumber, and poinsettia exhibited only toxicity to the 38 phosphonium compounds.

2:00. PORTER, W. L. and K. V. THIMANN, Harvard Univ., Cambridge 38, Mass. The molecular requirements for auxin action shown by modified indoles.

Consideration of the structure of a number of synthetic auxins has suggested that the primary growth action exerted by indole auxins depends upon the fractional positive charge of the —NH group and its distance from the anionic group (See Plant Growth Regulation, Iowa Univ. Press 1961, p. 444). A series of new indole compounds has therefore been prepared, in which the distance between the two charges and the intensity of the charge are modified. Among these compounds, (2-skatolethio)acetic acid has about 10% of IAA activity in the pea curvature test, while 2-Cl-indole-3-acetic acid methyl ester has bout 140%. The 2-Br ester has lower activity and the activities of other halogenated derivatives will be discussed. It is significant that the 2-Cl derivative is the first 2-substituted indole which is more active than the parent acid, and also significant that (2-skatalethio)acetic acid has higher activity than indole-2-acetic acid, since its —NH group is in a position spatially analogous to the ortho position of the active (2,4-dichlorophenylthio)acetic acid.

In indole compounds, it has been found that relative positive charge on the —NH group can be deduced from variations in the H-bonded —NH infra-red stretch frequency in the donor solvent, acetontrile. For substitutions on the benzene ring, —NH stretch has been found to vary linearly with Hammett sigma. Log of pea curvature activity is also a roughly linear function of stretch frequency and hence of sigma, providing that other factors (gross steric effects and lipid solubility of the nucleus) are relatively equal. It is concluded, therefore, that these facts provide some theoretical basis for auxin activity.

2:15. NANCE, J. F., Univ. of Illinois, Urbana, Ill. Effects of indoleacetic acid on CO_2 fixation and the oxidation of acetate by pea stem slices.

Indoleacetic acid (IAA) at 2.5 X 10^{-5}M promotes the oxidation of acetate-1-C^{14} by stem slices of etiolated pea seedlings. In concentrations of 2.5 X 10^{-4}M and higher, IAA is inhibitory. When the slices are in air containing 10% carbon dioxide, the rate of oxidation of acetate is enhanced over that for air but IAA becomes inhibitory at 2.5 X 10^{-5}M. Dark fixation of carbon dioxide-C^{14} by the slices is also inhibited by IAA concentrations of 2.5 X 10^{-5} and higher. It apeffect on some carboxylating enzyme of the cell, probably phosphorylenolpyruvate carboxylase.

2:30. WEDDING, R. T. and M. KAY BLACK, Univ. of California, Riverside, Calif. The response of oxidation and coupled phosphorylation in plant mitochondria to 2,4-dichlorophenoxyacetic acid.

Coupled mitochondrial prepartions show a reduced capacity for the formation of trichloroacetic acid-soluble organic phosphates in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D). Oxygen uptake by the mitochondrial is also influenced by 2,4-D, with the higher concentrations giving appreciable reductions in the rate of oxidation. A comparison of this effect using several TCA cycle acids and reduced diphosphopyridine nucleotide as substrates with mitochondria from different plant sources is given. The relatiion of this effect to external hydrogen-ion concentration and to constituents of the electron transport and phosporylation systems has also been investigated.

2:45. ALBERSHEIM, P., Harvard Univ., Cambridge, Mass. The inhibition of pectin trans eliminase by indoleacetic acid.

Indoleacetic acid (IAA) is known to enhance the synthesis of water soluble pectins at the expense of the water insoluble pectins. It is thought that this differentiation is mediated by the hormonal control of an enzymic balance. A pectinolytic enzyme has been found which is inhibited by physiological concentrations of IAA.

The enzyme, pectin trans eliminase, readily attacks the pectin chain molecule but has no effect on pectic acid. A study of the pectin breakdown products by ultraviolet absorption, ozonation, and reaction with thiobarbituric acid, indicated the presence of Δ-4,5 unsaturated galacturonic acid groups. The reaction seems to proceed by a trans elimination mechanism, initiated by the removal of the activated hydrogen at C-5. The glycosidic linkage in the β-position to the carbonyl function is then cleaved, resulting in a glycosyl anion and a double bond between C-4 and C-5. The reaction can be followed by measuring the absorption of ultraviolet light at 235 mμ resulting from the conjugation of the double bond with the C-6 carbonyl. The enzyme is inhibited 10% by $10^{-5}M$ IAA, 83% by $10^{-3}M$.

The enzyme has recently been crystallized. Studies are currently being carried out to ascertain its biochemical role and the mode of its interaction with auxin.

3:00. Recess.

3:15. WOLF, F. T., Vanderbilt Univ., Nashville, Tenn. and Carnegie Institution of Washington, Stanford, Calif. Mechanism of action of 3-amino-1,2,4-triazole.

Previous workers have shown that the effect of 3-amino-1,2,4-triazole (AT) in inhibiting growth of microorganisms may be reversed by adenine and/or histidine, and that AT inhibits riboflavin synthesis. In tomatoes, it has been reported that growth inhibition is reversible by purines, while inhibition of chlorophyll synthesis as well as growth inhibition may be reversed by riboflavin, FMN, or FAD.

Growth of *Chlorella pyrenoidosa*, strain 252, in Bristol's solution at 25° C. under continuous illumination in 5 per cent

CO_2 is inhibited approximately 50 per cent by 0.5 mg. AT per 100 ml. Growth of the alga was estimated by a Klett-Summerson photoelectric colorimeter equipped with a number 66 red filter. It was found that complete reversal of the growth inhibition could be induced upon the addition of 1.0 mg. per 100 ml. of adenine, guanine, hypoxanthine, or xanthine, and reversal was almost complete with this concentration of uric acid. No reversal was found with riboflavin, even in much higher concentrations.

With wheat seedlings grown in petri dishes in the laboratory, 10^{-4} M AT induces moderate inhibition of growth and pronounced inhibition of chlorophyll synthesis. Neither growth inhibition nor chlorophyll inhibition could be reversed by equimolar concentrations of adenine, guanine, hypoxanthine, xanthine, folic acid, xanthopterin, or histidine. Equimolar concentrations of riboflavin, FMN, or FAD were found to restore both growth and chlorophyll to normal or near normal levels. Findings essentially similar to those described for wheat have been obtained with tomato seedlings. It is concluded that in Chlorella AT affects a different system from that which is affected in wheat or tomato.

3:30. HABER, A. H., Biology Div., Oak Ridge National Laboratory, Oak Ridge, Tenn. Effects of indoleacetic acid on growth without mitosis and on mitotic activity in the absence of growth by expansion.

A given IAA treatment can greatly stimulate growth of the coleorhiza and epiblast while greatly inhibiting growth of seminal roots in young wheat seedlings. IAA produces these same effects on wheat seedlings growing without DNA synthesis, mitosis, and cell division after gamma-irradiation of the unsown grain. At different concentrations IAA can either increase or decrease mitotic activity during appearance of the first mitoses in thermodormant lettuce seeds, in which there is no over-all growth by expansion. IAA and kinetin exhibit a synergistic action on such mitotic activity, an action shown by the capacity of a given high concentration of IAA to inhibit mitotic activity in the absence of added kinetin and to increase mitotic activity in the presence of added kinetin. Thus IAA can have dual actions on growth in intact plants: it can influence (positively or negatively) growth by expansion apart from any possible actions on cell division, and it can influence (positively or negatively) mitotic activity apart from any possible actions on growth by expansion. High concentrations of IAA accelerate the deterioration of thermodormant lettuce seeds. These results are discussed in relation to several theories concerning the actions of auxin as a growth-regulator, as a herbicide, and a causative agent in the production of certain plant tumors.

3:45. ANDREAE, W. A. and M. W. H. VAN YSSELSTEIN, Pesticide Research Institute, Canada Agriculture, London, Ontario, Canada. Root growth inhibition following IAA and 2,4-D treatment.

Pea root tips were incubated for 16 hours in radioactive IAA or 2,4-D solutions and auxin accumulation and growth inhibition were studied. 2,4-D, but not IAA, accumulated in the tissues and growth remained inhibited after 2,4-D treatment but not after IAA treatment. Washing of 2,4-D treated tissues removed both the accumulated 2,4-D and the growth inhibition. The metabolic and physiological responses following IAA and 2,4-D treatment shall be discussed in the light of auxin herbicide action.

4:00. LEOPOLD, A. C. and S. L. LAM, Purdue Univ., Lafayette, Ind. The stem apex as a factor in auxin transport.

The ability of stems to transport auxin declines with increasing distance from the apex. Such a gradient is described for sunflower stems. Removal of the apex results in a rapid deterioration of the ability of the stem to transport auxin. If new lateral breaks are permitted to grow from the decapitated stem, the region just below the new apices is restored in the capacity to transport auxin. It is deduced that the apex plays a role in the maintenance of the ability of the stem to transport auxin. The implications of this factor in correlation effects and responses to decapitation treatments are discussed.

4:15. JACOBSON, ANN B. and B. J. ROGERS, Univ. of Chicago, Chicago, Ill., and Purdue Univ., Lafayette, Ind. Observations concerning the amino triazole effect on maize chloroplasts using the electron microscope.

Sub-lethal doses of amino triazole produce chlorosis in the basal portion of newly forming corn leaves. Since amino triazole does not appear to interfere with the biosynthesis of chlorophyll itself, studies were undertaken to determine whether the observed chlorosis would be attributed to a structural defect in the chloroplasts themselves.

Young corn seedlings were treated with amino triazole. Pieces of tissue from the first chlorotic leaf formed were fixed in unbuffered $KMnO_4$, and leaf sections were examined under the electron microscope. Large differences in the internal chloroplast structure were observed between control and treated plant material. The typical difference between chloroplasts of the vascular sheath and adjacent mesophyll were not apparent in the affected portion of the leaf. Further differences between control and treated materials will be discussed.

4:30. FOY, CHESTER L., Univ. of California, Davis. Calif. Detoxification of radioactive 2-chloro-4,6-alkylamino triazines by *Sorghum vulgare*.

Earlier investigations (Foy and Castelfranco, *Plant Physiol.* 35:xxviii, 1960; Castelfranco, Foy and Deutsch, Weeds, in press) showed that *Zea mays*, but not *Avena*, possesses a system that readily degrades simazine (2-chloro-4,6-bis(ethylamino)-s-triazine-2,4,6-C^{14}). The active constituent isolated from shoot extracts is capable of converting simazine-C^{14} to the corresponding 2-hydroxy analogue, which is non-herbicidal. The properties of the active constituent indicate that it is not a protein.

A similar detoxification mechanism for propazine (2-chloro-4,6-bis(isopropylamino)-s-triazine-2,4,6-C^{14}) evidently exists in *Sorghum vulgare* but not in *Avena*. Plants were grown in admixtures of soil and various concentrations of propazine-C^{14}. Samples were taken periodically from the time of imbibition of moisture by the seeds through death or maturity of the plants. Uptake, distribution and metabolism of the labeled compound were followed by radioautography, solvent extraction, counting and chromatography.

Both species absorbed and translocated the chemical equally well, initially. After 1-2 months sorghum was normal and the

shoots contained, on a dry weight basis, a maximum of 3.6 ppm propazine (includes C^{14}-containing, chloroform soluble metabolites). Oat plants similarly exposed contained 12.2 ppm and were all killed. Chromatography confirmed the presence of miner C^{14}-labeled substances other than propazine. With continued uptake of the chemical, C^{14} was distributed throughout the plants at maturity. Yet the levels of non-metabolized propazine in all tissues remained extremely low or became negligible, e.g. in seeds.

Recent studies with expressed sorghum sap and 90% acetone extracts thereof indicate, generally, that the triazine-destroying activities and other properties of the *Zea* and *Sorghum* systems are similar.

4:45. ASHTON, FLOYD M. and MING HWA FU, Univ. of California, Davis, Calif. Effect of ethyl N, N-di-*n*-propylthiolcarbamate on respiration and photosynthesis.

Mung bean seeds were surface sterilized with 0.1% $HgCl_2$ for ten minutes and soaked in sterile distilled water for twelve hours. The embryos were aseptically excised and transferred to 50 ml flasks containing 10 ml of 2% sucrose, inorganic salts, and various concentrations of EPTC (0, 10, and 100 ppm). They were placed in a shaking water bath at 30°C for 24 hours in the dark. Oxygen uptake by conventional Warburg techniques were 26.2, 33.4, and 45.2 μl/hr/100 mg fresh weight for 0, 10, and 100 ppm of EPTC, respectively. The fresh weight of the embryos was reduced by the EPTC treatments.

The rate of $C^{14}O_2$ fixation was determined for excised leaves of red kidney beans. The plants were grown in sand culture and treated with various concentrations of EPTC (0, 1, 10, and 100 ppm) in culture solution when they were ten days old. The EPTC treatment times were 5, 10, 50 and 100 hours. $C^{14}O_2$ fixation was inhibited at all concentrations of EPTC after 100 hours and at 100 ppm of EPTC after 50 hours; shorter exposure time did not inhibit $C^{14}O_2$ fixation.

The relative distribution of C^{14} the various labeled compounds following $C^{14}O_2$ fixation was determined for the 100 hour EPTC treatment time for all concentrations. This was determined by two-dimentional paper chromatography, autoradiography, elution of the radioactive spots, and subsequent counting. Although EPTC inhibited the rate of $C^{14}O_2$ fixation, it did not alter the relative distribution of C^{14} in the various labeled compounds.

SESSION F4. Room 201 Civil Engineering

GROWTH III

S. Gordon, *presiding*

1:30. SHROPSHIRE, W. Smithsonian Institution, Washington 25, D.C. Negative phototropism of *Phycomyces* sporangiophores to blue light stimuli.

The dioptrics in air of the cylindrical sporangiophore of Phycomyces Blakesleeanus confers on the distal side a focussing advantage of about thirty percent for unilateral blue stimuli. Data are presented in which this advantage can be nullified or reversed to produce negative curvatures by means of a rapidly diverging light stimulus.

Sporangiophores, while rotating, were adapted to continuous parallel blue radiant energy. (Intensity equivalent to 0.18 $\mu w/cm^2$ of monochromatic 440 mμ.) The adapting light was then cut off and the rotation stopped. Within thirty seconds a thin cylindrical glass lens was positioned to within 0.15 mm of the growing zone and with its long axis parallel to the long axis of the sporangiophore. A three minute blue stimulus at the same intensity was given and the lens removed. Reproducible negative curvatures were observed with a maximum of 8 to 13 degrees occurring within 7 or 8 minutes after the beginning of the stimulus. All experiments were done in a water saturated atmosphere to minimize avoidance responses due to the proximity of the lens. The data support Buder's conclusion that the focussing advantage is the principal effect which produces the light gradient necessary for phototropism. When compared to data obtained from sporangiophores immersed in liquids, the attenuation across the growing zone appears to be of the order of 10%. Therefore, under conditions in which the focussing advantage is less than 10%, negative curvatures are produced.

1:45. WIEGAND, O. F. and A. R. SCHRANK, Univ. of Texas, Austin, Texas. Effects of red light on *Avena* coleoptile growth.

The growth characteristics of the *Avena* coleoptile are modified by red light exposure. Elongation in the dark, is linear with time; in the light, the growth rate is greater and decays rapidly. These effects are evident in the intact seedling and in isolated sections. The characteristic growth patterns in the dark and in red light are independent of the indoleacetic acid concentration. The duration of growth of the coleoptile is limited by red light exposure, but its growth may be extended with exogenous growth hormones. The time and rate of emergence of the primary leaves are also influenced by exposure to red light. These relationships will be discussed.

2:00. KAUFMAN, P. B. and J. M. KATZ, Univ. of Michigan, Ann Arbor, Mich. Growth of node-internode segments of *Avena sativa* under red and far-red light regimes.

Node-internode segments excised from the P-1 node and internode (one node below the peduncle node) of *Avena sativa* shoots were surface-sterilized in 10% Clorox for 20 minutes and cultured on glass beads in 3 ml. 5% PO_4-buffered sucrose in 6 oz. culture bottles. Simple red (R) and far-red (FR) light sources were employed. Internodal extension in these segments was promoted in a red light regime and repressed in a far-red light regime. More elaborate experiments, using exposures to red and far-red light in 24 hour cycles, revealed promotion of internode growth in R, R-FR, R-FR-R, and R-FR-R-FR and repression of growth in FR, FR-R, FR-R-FR and FR-R-FR-R with least growth in the last series in each instance. Where gibberellic acid at 10 mg./l. was employed in the 5% buffered sucrose medium, the GA-augmented growth response was manifest under red light and abolished under the far-red treatment. The GA effect was repressed in reciprocal series of red and far-red light up to four cycles with greater growth repression occurring when far-red light was the initial 24 hr. treatment. The results reported here show that the type of light the segments receive in the first cycle is more important than that which terminates the series of cycles.

2:15. SISLER, E. C., W. H. KLEIN and REBECCA GETTENS, North Carolina State College, Raleigh, N.C. and Smithsonian Institution, Washington 25, D.C. The effect of red and far-red radiant energy and delta amino levulinic acid on the lag phase of chorophyll synthesis in bean seedlings.

The time course of chlorophyll synthesis at various stages of development on dark grown Black Valentine bean plants *(Phaseolus vulgaris)* was determined. Under the conditions grown, a lag phase does not occur until the beans are 6 days old. This might indicate that some material is depleted from either the leaves or cotyledons at about the 6th day. The rate of chlorophyll synthesis was increased by a pretreatment with red radiant energy (600-700 mμ) at all stages of development tested except in very old (23 days) leaves where the significance was doubtful. A subsequent treatment with far-red radiant energy (700-750 mμ) reversed substantially (40-100%) the effect induced by red energy at all stages of development tested.

Leaves infiltrated with delta amino levulinic acid synthesized larger amounts of material absorbing at 625 mμ in 80% acetone. Most of this material was not immediately converted to a material absorbing at 665 mμ in acetone (chlorophyll *a*). Chlorophyll synthesis occurs without a lag phase in leaves infiltrated with delta amino levulinic acid during the first hour of subsequent irradiation. A pre-induction with red energy does not increase chlorophyll synthesis in delta amino levulinic acid infiltrated leaves. Beyond an hour, the chlorophyll synthesis declines somewhat at low light intensities and the chlorophyll synthesized appears to be destroyed by high light intensities.

2:30. DOWNS, R. J., U.S. Dept. of Agriculture, Beltsville, Md. Photocontrol of anthocyanin synthesis in dark-grown milo seedlings.

Two photoprocesses control anthocyanin synthesis in dark-grown milo seedlings. The first photoreaction requires high energies and has a maximum response between 470 and 480 mμ, with little or no action at wavelengths longer than 600. The second photoreaction is the low-intensity, red, far-red reversible one that operates through phytochrome.

A brief period of far-red nullifies the effect of the initial, high-intensity photoreaction by preventing completion of one of the reactions that ultimately leads to anthocyanin formation. This far-red block can be imposed at any time after the initial photoreaction and stop all the product that has not passed the point of blocking. The far-red inhibition is reversed by irradiation with red and reimposed by another exposure to far-red. The stability of the product behind the far-red block was measured by allowing various durations of darkness to intervene between imposition of the far-red and removal of the block by irradiation with red. About 4 hours was required for disappearance of half the product.

2:45. IKUMA, HIROSHI and K. V. THIMANN, Harvard Univ., Cambridge 38, Mass. Effects of nitrogen atmosphere on the germination of photosensitive lettuce seeds.

When seeds are temporarily subjected to a nitrogen atmosphere during light treatment, the red/far-red reversible pigment system functions normally, and the shape of the dose-response curve does not differ between nitrogen-treated seeds and air controls. This suggests that the pigment conversion is a pure photoreaction.

If far-red light is given at progressively longer intervals after red, it is well known that the seeds gradually escape from its inhibitory effect. This rate of escape is shown to be dependent on temperature. It does not take place during nitrogen treatment, but commences as soon as the seeds are returned to air.

Short periods in nitrogen immediately after red irradiation greatly reduce the final per cent germination of the seeds, whereas, at a later stage, comparatively long periods in nitrogen cause only slight inhibition.

These observations suggest that the pigment conversion must be followed closely by an oxidative reaction which requires the presence of the physiologically active form of pigment, P_{fr}, and probably of molecular oxygen. The inhibitory effect of nitrogen, when given directly after red irradiation, may be ascribed to a dark conversion of P_{fr} to P_r. Such seeds were found to germinate eventually in light.

Thus, in lettuce seed germination, at least four phases can be distinguished; (1) imbibition of water which activates subsequent stages, (2) induction by red light—a pure photoreaction, and (3) dark processes, beginning with an oxidative reaction, which lead to (4) visible protrusion of the radicle.

3:00. Recess.

3:15. SIEGELMAN, H. W., E. M. FIRER, W. L. BUTLER and S. B. HENDRICKS, Plant Industry Station, Beltsville, Md. Partial purification of phytochrome.

Phytochrome (P) can be extracted in dilute alkaline buffer from 1-kg lots of 6-day-old dark-grown barley seedlings, frozen at −78° and ground in a single-end runner mill. The activity of the photochromic pigment is determined by differential spectrophotometry. Currently, the ground material is extracted in two volumes of .008 M NaOH followed by adsorption on DEAE. The DEAE column is washed with a large volume of .025 M potassium phosphate at pH 7.5 and then eluted with 0.25 M potassium phosphate at pH 7.5. The eluate is concentrated by dialysis against polyethylene glycol (20,000 MW). A 4-fold purification and a 50-fold concentration over the original extract are attained in 24 hours. All operations are performed at 2° and an -SH compound is required at all time for stability. The solution contains the P_{660} - P_{730} photoreversible forms. It is stored in 30-percent ethylene glycol at −15°, which can be removed by gel filtration on Sephadex G-50 prior to further procedures.

A bluish-green precipitate showing obvious photoreversible changes in color appears after the concentration dialysis and centrifugation. Spectroscopic examination of the precipitate shows photoreversible absorption maxima near 660 mμ and 710 mμ after far-red and red irradiations, respectively. A partial solubilization of the precipitate yields a clear-blue solution. Heating the precipitate to 55° yields a non-reversible form with an absorption maximum near 680 mμ.

3:30. BUTLER, W. L., H. W. SIEGELMAN and S. B. HENDRICKS, Plant Industry Station, Beltsville, Md. Some photochemical properties of phytochrome.

The action spectra for the photochemical interconversion of the two forms of phytochrome $P_{660} \gtrless P_{730}$ has been determined. The kinetics are first order in both directions. Partially purified solutions of phytochrome were irradiated with known energies of monochromatic light on the Beltsville spectrograph in order to determine the rate constants for both directions of the photoreaction as a function of wavelength. The product of the molar extinction coefficient, α, and the quantum yield of the photoreaction, ϕ, can be calculated from the first order rate constants. The plots of $\alpha\phi$ vs λ for both forms of phytochrome are direct measures of the absolute absorption spectra. The data show that the quantum yield of the forward direction is greater than that of the reverse.

Fluorescence emission from P_{660} but not from P_{730} has been observed. Fluorescence excitation spectra show the excitation maximum is at about 665 mμ. In many cases the fluorescence excitation spectra provide a more sensitive indication of the absorption band of P_{660} than absorption spectra. The presence of phytochrome can be demonstrated in a dark grown bean leaf by fluorescence excitation spectra at —196° C. whereas it is not detected in the absorption spectrum of the leaf. The fluorescence excitation spectra of solutions of phytochrome which have been stored several months at low temperature show an irreversible band at 680 mμ which was not present initially. The appearance of this band is associated with a loss of the reversible band at 665 mμ and probably represents a denatured form of phytochrome.

3:45. BONNER, B. A., Yale Univ., New Haven, Conn. Properties of phytochrome from peas.

The purification and characterization of phytochrome has been continued. Preparations showing a ratio of absorbancy at 400 mμ to that at 600 of less than one show a region of reversible change in absorption spectrum centering around 415 mμ accompanying the larger changes at 660 and 735 mμ induced by red and far-red irradiation. The far-red absorbing form absorbs more at 415 mμ than does the red absorbing form. Blue light (410 mμ) will bring about the same conversion as does far-red. These preparations are blue-green in color.

Available evidence indicates that the loss in the magnitude of reversibility which occurs on prolonged storage at 2° or more rapidly at room temperature is accompanied by a loss of total absorption in the red region. Intermediate stages in the inactivation exist which have an altered response to light in which the changes in absorption at 660 and 735 mμ have been partially uncoupled. Urea causes the disappearance of reversibility and alters the absorption spectrum providing further evidence that the reversibility is intimately linked with the intact state of a protein component of the pigment. Evidence bearing on the possible nature of the chromophore will be discussed.

4:00. SOROKIN, C. and R. W. KRAUSS, Univ. of Maryland, College Park, Md. The effects of light on cell division.

Cell division was studied in suspensions of the high-temperature strain of *Chlorella pyrenoidosa* in which cell division was separated in time from growth by a synchronization technique. Synchronization was achieved by an intermittent light-dark regime.

It was found that the time of incipient cell division, the rate of cell division, and the number of daughter cells per one mother cell, depend on light intensity and on the length of light period, as well as on temperature during both growth and cell division periods. At a given temperature all these characteristics are generally favored by the increase in light intensity, but only to a certain illuminance beyond which an increase in light intensity inhibits cell division. The effect of the length of the light period depends on light intensity and on temperature. The increase in temperature to a certain limit during both light and dark periods usually favors cell division.

4:15. POWELL, R. D. and M. M. GRIFFITH, Univ. of Florida, Gainesville, Fla. The effect of γ-radiation on red light induced growth of bean leaves treated with kinetin and gibberellin.

Disks were cut from the primary leaves of dark-grown bean seedlings and placed on filter paper saturated with a KNO_3-sucrose solution. Some disks were treated by adding either gibberellin or kinetin to the solution on the filter paper. All manipulations were done in complete darkness. Further treatment included exposing the disks to varying amounts of γ-Radiation and to red light. After an appropriate period of time the diameter of each disk was measured and the mean cell diameter was determined. Measurements of disks indicated the following:

1. Exposure to red light causes increased rate of growth
2. Exposure to 10 kr γ-radiation reduces growth of untreated disks
3. All effectiveness of red light as a growth promoter is removed by exposure to 10 kr γ-radiation
4. Following exposure to 10 kr γ-radiation red light treated disks and untreated disks showed comparable growth
5. Kinetin stimulates growth of disks and exposure to 10 kr γ-radiation produced no appreciable inhibition of this growth
6. Gibberellin stimulates growth of disk and this stimulation is reversed by γ-radiation.

Since cell division is more sensitive to γ-radiation than is enlargement, it is concluded that stimulation of growth by red light and gibberellin is brought about by increased cell division. The kinetin induced growth is primarily an effect on cell enlargement.

4:30. LOERCHER, LARS, Smithsonian Institution, Washington 25, D.C. The mode of action of cobalt in the expansion of etiolated leaf tissue.

The expansion of dark-grown leaves is markedly promoted by exposure to red light. An additional growth stimulation can be exerted by cobalt ions (Co^{++}), which also promote growth in the dark.

Etiolated bean leaf disks were incubated in a nutrient solution in the dark for 48 hours. The optimal concentration of Co^{++} was 3×10^{-4}M. The metabolic role of Co^{++} was studied by use of specific inhibitors.

2,4-dinitrophenol (DNP), which uncouples oxidative phosphorylation, inhibits the growth of leaf disks, but the growth promotion due to Co++ is not impaired by DNP.

Adenosine triphosphate (ATP) levels were determined in leaf tissue after treatment with DNP and/or Co++. It was found that Co++ by itself does not affect the amount of ATP appreciably, whereas with DNP the ATP level drops to less than 10% of the control. If Co++ is added with DNP, no decrease occurs in the amount of ATP.

It appears that this effect is not due to the formation of a complex between DNP and Co++ which is inactive in the oxidative phosphorylation process.

Experiments with isolated mitochondria indicate that the effect of DNP on the incorporation of inorganic phosphate is only partially balanced by the addition of Co++.

4:45. KOHL, HARRY C., Jr., Univ. of California at Los Angeles, Los Angeles 24, Calif. Development of orchid protocorms under environments varied with respect to orientation, submersion and light.

Cymbidium orchid protocorms on agar, in light, the normal situation, branch for a time from the base forming new protocorms which may eventually form an apical bud which in turn becomes a shoot. Adventitious roots initiate on the shoot resulting in a complete plant. Protocorms in submerged culture form many new protocorms but these do not form shoots. Protocorms in shake culture proliferate for a time and then die. Protocorms grown in the dark lose their chlorophyll. They also form new protocorms which initiate roots. Protocorms grown in a variety of different quality lights showed large quantitative growth responses but few qualitative differences. Sliced or crushed protocorms frequently generate new protocorms. All experiments were performed in sterile culture with only inorganic additives except for sucrose and, in some instances, agar.

5:00. DUGGER, W. M., Jr., and O. C. TAYLOR, Univ. of California, Riverside, Calif. Interaction of light and smog components in plants.

Evidence will be presented to show that the photochemically produced atmospheric oxidant, peroxyacetyl nitrate (PAN), interacts with some plant constituent produced in the light to cause damage. This interaction of PAN and the plant, resulting in damage, is also contingent upon light during exposure to the oxidant as well as after exposure. A dark period at any one of these times will protect the plants from oxidant damage. The study shows that the oxidant-photochemical relationship affects the photosynthetic reactions. Oxygen evolution and TPN reduction from chloroplast preparations are reduced by PAN exposure.

Ozone, another oxidant produced photochemically in the atmosphere, interacts with plants in a different manner than PAN. A 48 to 72 hour dark period prior to exposure to this oxidant is necessary to prevent plant damage. Damage is associated with the endogenous carbohydrate level; plants depleted of carbohydrates by a long dark period are made susceptible to ozone damage by artificially increasing the sugar content.

In all experiments where plants are exposed to darkness prior to the oxidants, the stomates were observed to be open at the time of exposure. A resistance porometer and leaf cup were used to measure stomatal opening.

This evidence does not favor the hypothesis that stomates are the controlling factor in smog damage to plants; rather it is the photochemical reaction between the oxidants in smog and plants. This in part explains the inconsistencies in past observations that have been made regarding oxidant damage to plants.

SESSION F-5. Room 201, Civil Engineering

ANNUAL BUSINESS MEETING, PHYSIOLOGICAL SECTION, BOTANICAL SOCIETY OF AMERICA

Immediately following Session F-4.

J. Torrey, *presiding*

THURSDAY, AUGUST 31

THURSDAY MORNING
CONCURRENT SESSIONS G1, G2, G3, G4

SESSION G1. Room 4, Memorial Center

***Informal Discussion:* METABOLIC CONTROL MECHANISM**

Robert Stanley, *presiding*

Participants: JOHN A. ROMBERGER, FRANK E. LOEWUS and JOHN C. GERHART

8:30.

SESSION G2. Room 318, Memorial Center

BIOCHEMISTRY IV

G. R. Noggle, *presiding*

8:30. MITCHELL, R. L. and I. C. ANDERSON, Iowa State Univ., Ames, Iowa. Inactivation of catalase in corn and bacteria by visible light.

The catalase activity in carotenoid mutants of corn (W_3) and bacteria *(Sarcina lutea)* is reduced when exposed to bright light. Catalatic activity is retained when these mutants are

placed under anaerobic conditions. This retention is most distinct in corn. In the presence of dilute concentrations of ethanol and methanol, catalatic activity is also maintained. The catalatic activity of normal corn and bacteria is not appreciably affected by exposure to bright light.

Inactivation of crystalline catalase by light is pH dependent. Activity is retained in the presence of ethanol, methanol or hydrogen peroxide, but these treatments cause no change in the absorption spectra of catalase. An action spectrum of catalase destruction coincides with its absorption spectrum.

The relationship between these light-air-chemical interactions on porphyrin structures and the possible protective value of certain naturally occurring substances, such as the carotenoids, will be discussed.

8:45. WEINSTEIN, LEONARD H., Boyce Thompson Institute for Plant Research, Inc., Yonkers, N.Y. Effects of atmospheric fluoride on metabolic constituents of tomato and bean leaves.

Time-course experiments were run in order to determine the effect of fumigation with HF (12.4 and 1.3 p.p.b., v/v) on dry weight, fluorine content, and levels of free amino acids and amides, nonvolatile organic acids, free sugars, ribo- and deoxyribonucleic acids, and chlorophylls *a* and *b* in leaf tissues of Bonny Best tomato and Tendergreen bean plants. During fumigation plants were harvested at 3-day intervals for 9 days. This was followed by a recovery period during which plants were again harvested at the same intervals.

The total F content of both tomato and bean leaf tissues decreased during the recovery period. The free sugar content of leaves was reduced in both species during the fumigation and recovery periods. In general content of nonvolatile organic acids increased during the first part of the fumigation. This was followed by decreasing levels of acids during the rest of the fumigation period. During the recovery period, the acid content increased, and often reached levels higher than in the controls.

The length of fumigation and its influence on the results obtained will be discussed.

9:00. MILLER, ERSTON V. and STEPHEN E. OLAH, Univ. of Pittsburgh, Pittsburgh 13, Pa. Some physiological studies on the effect of low temperatures on the fruits of the tomato *(Lycopersicon esculentum)*.

It has been known for some time that prolonged storage of tomatoes at low temperatures (0° to 10° C) may produce a physiological disorder known as low-temperature injury or chilling. The disorder may be characterized by development of a watery texture, a failure to ripen with the characteristic tomato color or by development of decay. There is at present very little information on the fundamental changes which occur in tomato fruits when they develop low temperature injury.

In the investigations now being reported, green tomatoes were stored at 0° C and 5° C for varying periods of time. At the end of the particular storage period half of the sample was analyzed immediately while the other half was ripened before being analyzed.

Storage for 10 days at 5° C reduced the amount of ascorbic acid which developed during ripening and 20 days of storage practically inhibited production of ascorbic acid.

Similar studies on the iodine-reducing value of the juice of the fruit showed an even greater reduction of this activity after prolonged storage.

Phosphatase activity was higher in ripe, unstored fruits than in the green ones. Storage at 5° C for 20 days reduced phosphatase activity and 10 days at 0° C resulted in lower phosphatase activity in the ripened than in the green tomatoes.

Additional studies were conducted on pigment content of tomatoes which had been stored at 5° C for 25 days. Several of these lots lent themselves to more detailed study because of differences in ripening rate. Accordingly when one-fourth of the individuals were "red ripe," the others were separated into "pink," "turning" and "green" groups. Pigments were extracted and an absorption spectrum was determined for each group. The pigment from red ripe and pink fruits showed a maximum absorption at 470 millimicrons; for turning fruits it was 460; whereas in the green fruits the maximum shifted into the violet; i.e., 420 millicrons. It is concluded that chilling of green tomatoes may exert a direct effect either upon formation of lycopene or on one of its precursors.

9:15. ANDERSON, D. G. and J. W. PORTER, VA Hospital and Univ. of Wisconsin, Madison, Wis. Biosynthesis of phytoene and squalene by plant enzyme systems.

Results subsequent to the recent report from this laboratory, (Anderson, D. G. and Porter, J. W., *Fed. Proc.* 20:350, 1961) have shown that the biosynthetic system for the formation of phytoene may be solubilized by detergents. Furthermore, ammonium sulfate precipitated protein fractions of the solubilized system are also active in the biosynthesis of phytoene.

The "unidentified colorless polyene" reported to arise on injection of 2-C^{14}-mevalonic acid into ripening tomatoes (Anderson, D. G., Norgard, D. W., and Porter, J. W., *Arch. Biochem. Biophys.* 88:68, 1960) is also formed by the carrot plastid system. The major portion of this labeled material arising from both sources has been tentatively identified as squalene by gas chromatography, adsorption chromatography, and hexahydrochloride derivative formation. In both cases the incorporation of radioactivity into squalene is much greater than the incorporation into phytoene. C^{14}-labeled acid labile terpenol pyrophosphates, whose major component is farnesyl pyrophosphate (80-90%) (Anderson, D. G., Rice, M. S. and Porter, J. W., *Biochem. Biophys. Res. Comm.* 3:591, 1960. serve as precursor for squalene in the carrot plastid system.

While the phytoene biosynthetic system, in main part, survives solubilization and ammonium sulfate precipitation, the squalene biosynthetic system is lost during such treatment. Additional characteristics of each enzyme system will be reported and discussed.

9:30. CHING, T. M., Oregon State College, Corvallis, Oregon. Fatty acids in Douglas fir seeds.

Douglas fir seeds contain 40-45% fatty substances, of which 70% is glycerides, 7% acetone precipitable phospholipids, 3% free fatty acids, and 1% non-saponifiable material.

The fatty acid components from glycerides and free fatty acid fractions were separated and estimated by gas chromatography. A maximum number of 24 components were found in some cases. The carbon chain length varied from C_6 to C_{22} with linoleic, oleic, palmitic and stearic as the major components. In general, the same number of components were found in both fractions in a given lot of seed. Percent distribution of shorter and more saturated fatty acids was higher in the free fatty acid fraction than in the glyceride fraction. Fatty acids with odd numbered carbon chain were observed, although in small quantity.

9:45. WOLF, F. T., J. G. CONIGLIO and J. T. DAVIS, Vanderbilt Univ., Nashville, Tenn. The fatty acids of spinach chloroplasts.

Chloroplasts of spinach were isolated by homogenization and differential centrifugation in 0.35 M NaCl, saponified by treatment with alcoholic KOH, and non-saponifiable substances were removed by multiple extractions of the alkaline solution with petroleum ether. The solution was then acidified, and the fatty acids were extracted in petroleum ether. Aliquots of the extract were treated under nitrogen with diazomethane to esterify the fatty acids. Fatty acid composition was determined by gas chromatography of the methyl esters in a Barber-Colman instrument, using a column packed with 12.5 per cent of ethylene glycol-succinate polyester coated on acid-washed 80-100 mesh chromosorb W.

The components were identified by comparison of the retention times with known standards, and the amount of each component present was determined by integration of the area under the curve. An aliquot of the methyl esters was hydrogenated using platinum oxide at a catalyst, and the hydrogenated fatty acids were also analyzed by gas chromatography. The agreement of analytical data on the unhydrogenated and hydrogenated samples was satisfactory.

The fatty acid composition was determined as 68.9 per cent linolenic acid, 11.2 per cent palmitic acid, 10.8 per cent of a compound tentatively identified as a 16-carbon trienoic acid, 4.6 per cent linoleic acid, 3.5 per cent palmitoleic acid, and 1.1 per cent oleic acid. At least five other fatty acids were detected in trace quanities. About 89 per cent of the total fatty acids were unsaturated, and approximately 75 per cent of the total fatty acids were of the 18-carbon series. The possible significance of these findings is discussed.

10:00. Recess.

10:15. NANDI, D. L. and E. R. WAYGOOD, Univ. of Manitoba, Winnipeg, Manitoba, Canada. Enzymes of porphyrin synthesis in wheat leaves.

The following enzymes involved in the biosynthesis of porphyrin have been demonstrated in wheat leaf extracts (a) Succinic thiokinase catalysing the formation of succinyl CoA (b) an enzyme that catalyzes the condensation of succinate and glycine to Δ-aminolevulinic acid (ALA) (c) ALAse catalyzing the formation of porphobilinogen (PBG) and (d) PBG-D catalyzing the deamination of PBG and its condensation to a porphyrin type compound. The important points in reaction systems will be discussed.

10:30. YAGI, T. and A. A. BENSON, The Pennsylvania State Univ., University Park, Pa. Sulfolipid metabolism: lysosulfolipid formation.

The plant sulfolipid is a sulfoglycosyl diglyceride. It contains 6-sulfo-α-D-quinovopyranosyl-(1→1′)-D-glycerol and two fatty acids. Its two fatty ester groups were recognized by deacylation of the C^{14}-labeled sulfolipid to yield 34-36 units of C^{14} fatty acids.

Sulfolipid-S^{35}, isolated by paper chromatography was incubated with a *Scenedesmus* extract prepared by grinding the cells with alumina. The sulfolipid was hydrolyzed to form sulfoquinovosyl glycerol and an unknown (X) which had R_f values slightly less than those of the sulfolipid both in phenol-water and butanol-propionic acid-water solvent systems. The substance X was organic-soluble and yielded sulfoquinovosyl glycerol upon hydrolysis either by methanolic potassium hydroxide or enzymatically by treatment with the *Scenedesmus* extract. These facts suggest that the substance X has but one fatty acid in its glycerol moiety, and should be termed a lysosulfolipid, the analog of lysolecithin.

While the cell-free extract of *Scenedesmus* catalyzed the hydrolysis of both sulfolipid and phosphatides, snake venom lecithinase preparations failed to attack the sulfolipid, indicating the presence of a specific *sulfolipase* in algae. (This work was supported by the National Science Foundation and The Pennsylvania Agricultural Experiment Station.)

10:45. NG, MAVIS Y. L., K. V. THIMANN and SOLON A. GORDON, Harvard Univ., Cambridge, Mass. and Argonne Laboratory Atomic Energy Commission, Chicago, Ill. The action spectrum for anthocyanin formation in *Spirodela oligorrhiza*.

Anthocyanin formation, as has long been known, is strongly promoted by light, but high energies are required. The availability of a very high energy-high dispersion spectrum made it possible to explore the action of different wavelengths on this system. It was found best to hold the plants 40 hours in the dark after exposure, in order to allow maximal development of the pigment. The effectiveness of different wavelengths was then found to vary widely; for example, at 700 mμ, an irradiance of about 20 kiloergs of energy per sq. mm. is enough to saturate the anthocyanin forming system, but at 400 mμ, no saturation was detected up to 1500 Kergs/sq. mm. By varying the dose rate in different regions along the spectrum, points of maximum efficiency were found in the short ultra-violet and at 700 mμ. Although sugar and riboflavin are known to promote anthocyanin synthesis in Spirodela, the action spectrum bears no relation to the peaks of photosynthesis or to the absorption spectrum of riboflavin. The peak at 700 mμ agrees with that found by others for red cabbage, but data in the ultra-violet have not yet been presented by other workers. The 700 mμ band does not appear to derive from fluorescence emission following excitation at the 300 mμ peak. Emission spectra of fractions extracted from the plants by various solvents support this inference.

11:00. KURAISHI, SUSUMU, J. C. GARVER and F. M. STRONG, Univ. of Wisconsin, Madison, Wis. Mass culture of *Physarum polycephalum* plasmodium and purification of its yellow pigments.

Shake flask cultures of *Physarum polycephalum* Schw. (strain M3) grown on the medium of Daniel and Rusch (*J. Gen. Microbiol.*, in press, 1961) yielded 5 to 7 g. of plasmodium (dry weight) per 1. of medium. Three main pigments having peaks at 418, 379 and 360 mμ, (designated A, B, and C, respectively) were extracted by 80% acetone. After removal of the acetone, the aqueous solution was brought to pH 1.5 by conc. HCl. The resulting red-orange precipitate was washed by water and Skellysolve C, and dissolved in absolute acetone. The acetone-soluble pigments were chromatographed on acid-activated Florisil, heat-activated Florisil, DEAE cellulose and CM cellulose. In concentrated solution pigment A was readily converted into B, and B into C. Pigment A had a peak at 418 mμ, showed no fluorescence, no peak at 280-240 mμ, and yielded no amino acids after hydrolysis. The best preparation showed: absorbancy index 1200 (acidic methanol, 420 mμ, c = 1 g. per 1.), C 60.08%, H 5.98%, N 3.08%, ash 2.40%, S and Fe nil. The pigment thus is neither a pteridine as proposed by Wolf (Photoperiodism and Related Phenomena in Plants and Animals, 321, 1959, Washington, D. C.) nor a peptide type pigment as suggested by Dresden (Ph.D. thesis, University of Wisconsin, 1959). After preliminary work with 15 1. fermentations, a 150 1. fermentation run in a 50 gal. Pfaudler tank with minimum agitation yielded 290 g. (dry weight) of plasmodium after 5 days. This indicates that large scale culture of this organism is feasible.

11:15. SMITH, D. H., Jr., F. E. MUMFORD, J. E. CASTLE, Experimental Station, E.I. du Pont de Nemours and Co., Wilmington 98, Del. IAA oxidase inhibitor from etiolated pea epicotyls.

Hillman and Galston have shown that the indoleacetic acid oxidase activity of the buds of etiolated pea plants is inhibited by red light (ca. 640 mμ) and that the effect is reversed by near infrared (730 mμ) light. They presented evidence that the inhibition is due to increased concentration of a dialyzable inhibitor. Investigation of the structure of this IAA oxidase inhibitor has revealed that it is a derivative of kaempferol. Although kaempferol and related flavanoids have been found previously in many plant species the role that these compounds play in the biochemistry of the plant has remained obscure. The finding here that a derivative of kaempferol is an IAA oxidase inhibitor suggests that flavanoids may exert an effect on plant growth through their action on the IAA oxidase system.

11:30. FURUYA, M., A. W. GALSTON and B. B. STOWE, Yale Univ., New Haven, Conn. Isolation of cofactors and inhibitors of indoleacetic acid oxidase from pea plants.

Natural inhibitors of indoleacetic acid (IAA) oxidase have been reported in green pea tissue (Tang & Bonner, 1948), and in red light pretreated etiolated buds (Hillman & Galston, 1957). Natural cofactors have been detected in dark grown buds and roots (Sharpensteen & Galston, 1959). The chemical nature of these compounds has, until now, been obscure.

We have isolated these active substances from green pea leaves and etiolated pea plumules and have partially identified them as at least four flavonol derivatives. Two quercetin glycosides found only in light-grown peas act as natural inhibitors, while two kaempferol derivatives detected are natural cofactors. Two of these four appear to be new natural compounds, yielding a flavonol, a phenyl propanoid, and a sugar after hydrolysis, while the other two compounds seem to be common flavonol glycosides. The production and distribution of cofactors and inhibitors of IAA oxidase vary with the organ and are affected by light conditions under which the pea seedlings are grown.

In a standard reaction mixture consisting of peroxidase, IAA, and phosphate buffer, 50% inhibition of IAA oxidation occurred when 3×10^{-6}M quercetin-3-glucoside was added, whereas the optimal promotive concentration of natural cofactor, such as p-coumaryl kaempferol triglucoside, was 5×10^{-6}M. This corroborates previous reports that IAA oxidase is activated by monophenols, and inhibited by diphenols (Goldacre et al., 1953).

11:45. SURREY, K., Argonne National Laboratory, Argonne, Ill. Spectral response of phosphate metabolism in germinating lettuce seeds.

Seed germination is an endergonic system and the energy so expended is evidently derived from the reserve food material. Of the known physiological processes involved in the energy transformation, phosphate metabolism occupies a central position and as such must play an important role in the germination process. Lettuce seed germination has repeatedly been demonstrated to be influenced by visible light. Coupled with the above two lines of approach it is postulated that if radiant energy effects seed germination, it must have an analogous influence on endogenous phosphate metabolism and must alter the metabolic rates, at the molecular level, which is ultimately manifested in structural development.

Consistent with this reasoning, the action spectra (determined at various stages of germination) repeatedly indicated a maximum potentiation (of P^{32}-orthophosphate uptake, Pi, and its esterification, Po) between 550 and 650 mμ a maximum supression at 750 mμ and a partial suppression at 475 mμ. A slight stimulation was again encountered at 400 mμ, a stimulation which was then checked by shorter wave lengths of the ultra-violet spectrum.

Photoactivation of phosphate metabolism, by the three loci (550, 600 and 650 mμ) of potentiating spectral band, was found to be reversed by far red (750 mμ) light, and vice versa, in an alternating sequence. A complete reversibility depended entirely upon a fixed energy-dose relation between the two counteracting wave lengths and differed considerably from the other pairs.

When the hydrated seeds were continuously irradiated, red light greatly promoted phosphate metabolism, far red suppressed, and the dark-grown seeds exhibited an intermediate activity between the two extremes.

Light controlled phospholipid metabolism will also be discussed.

SESSION G3. Room 302, Memorial Center

PHOTOSYNTHESIS IV

J. B. Hanson, *presiding*

8:30. HONDA, S., T. HONGLADAROM and S. WILDMAN, Univ. of California, Los Angeles, Calif. Mitochondria and chloroplasts in living plant cells. (film)

This color movie, taken at normal speed, will illustrate the behavior of living spinach mesophyll cells as observed by phase microscope at magnifications up to 1250 diameters. Mitochondria and sphaerosomes are revealed with great clarity as they stream in the cytoplasm. Of particular interest is the behavior of a jacket of mitochondrial material surrounding each chloroplast because the jacket forms protuberances which may extend for several chloroplast diameters into the cytoplasm, at which point the protuberances can segment into individual mitochondria. The effect of 2,4-dinitrophenol on protoplasmic structures will be shown wherein the mitochondria are caused to change shape, and then regain their original shape when the uncoupling agent is removed. In addition to photosynthesizing spinach cells, other pictures will show that slight pressures exerted on living tobacco hair cells will cause mitochondria to change shape from elongated to spherical particles, the change being reversible when the pressure is removed. Some sequences of streaming protoplasm in potato tuber cells will also be shown.

8:45. HONGLADAROM, T., S. HONDA and S. WILDMAN, Univ. of California, Los Angeles, Calif. Observations on mitochondrial-like substance enveloping chloroplasts in spinach mesophyll cells.

Much of the organization of the protoplasm in living cells can be resolved by phase microscopy. Although the chloroplasts remain stationary the envelope of optically-dense cytoplasm surrounding the individual chloroplasts is in constant motion giving rise to protuberances which may extend to several chloroplast diameters. The constantly moving envelope is distinct from kinoplasm and shows marked absorption of light passing through a 550 mμ interference filter, as do the mitochondria. The protuberances may segment into particles which appear identical to mitochondria and the mitochondria sometimes coalesce with the chloroplast envelope following which they no longer appear as mitochondria. Buffered OsO_4 initially stimulates cytoplasmic streaming and causes a marked increase in the amount of visible sphaerosomes and a marked decrease of mitochondria. The effect of buffered OsO_4 does not seem to be especially deleterious to structures but the disorganization of tissue infiltrated with OsO_4 followed by sectioning indicates that the fixed state is more easily disrupted by mechanical means. The considerable quantity of mitochondria and large amounts of cytoplasm surrounding the chloroplasts which gives rise to mitochondrial-like particles contrast with views that there are very few mitochondria in special photosynthetic cells in leaves. It is possible that ATP is formed in photophosphorylation not in the chloroplasts themselves but in the enveloping layer of cytoplasm. This layer of of cytoplasm may not act biochemically as mitochondria since, e.g. DNP does not affect the envelope as it does the mitochondria. These observations do not necessarily contradict hypotheses concerning the self-sufficiency of chloroplasts to carry out photophosphorylation but opens the way for further interpretations.

9:00. GRANICK, S., Rockefeller Institute, New York City. Localization of the enzymes of the porphyrin-chlorophyll biosynthetic chain in plastids and mitochondria.

End products of this biosynthetic chain are the iron porphyrins and their derivatives (as cytochromes) in plastids and mitochondria; and the magnesium porphyrins (as chlorophylls) in plastids. Attempts have been made to determine which enzymes of this chain are localized in these bodies as a part of a study of cytoplasmic inheritance and differentiation. Difficulties in these determinations will be discussed.

In animal mitochondria only two of the six enzymes involved in protoporphyrin synthesis appear to be present in the mitochondria (Sano & Granick *J.B.C.* 236, 1173, 1961).

In plastids the following experiments suggest that most if not all the enzymes involved in chlorophyll biosynthesis are contained in them:

1. Mature tobacco plastids convert δ-amino levulinic acid (δAL) to porprobilinogen. 2. Etiolated barley and bean plastids convert δAL to protochlorophyllide (MgVP). In barley the plastid center contains carotinoids and MgVP. By fluorescence microscopy, after treatment of etiolated leaves with δAL, the porphyrins identified within the proplastids are protoporphyrin (PROTO), Mg PROTO, and MgVP. Thus the enzymes that convert PROTO to MgVP are considered to be localized in the proplastids. 3. Streptomycin-bleached Euglena cells grown in the dark contain no detectable enzymes of this chain. After growth of these cells in the light, enzymes appear that convert δAL to coproporphyrin and to PROTO. These porphyrins are seen to be localized in colorless plastids, 2 μ diam., by fluorescence microscopy. 4. The accumulation of Mg PROTO from δAL in etiolated barley and bean leaves is stimulated better by pyridyl-2-aldoxmine than by α, α-dipyridyl. These investigations were supported in part by NIH RG-4922 and by NSF G-14418.

9:15. BERGERON, J. A. and R. M. SMILLIE, Brookhaven National Laboratory, Upton, L.I., New York. Some aspects of the fine structure of the blue-green alga, *Anacystis nidulans*.

The pronounced tendency of the phycocyanins and Blue-Green Algae to be released as soluble proteins would seem to indicate that the photosynthetic activity of these auxiliary pigments does not depend upon fixed structural relationships with the chlorophyll-containing components. This view is consistent with the reports of photophosphorylation in cell-free extracts, devoid of phycocyanin; nevertheless, it has also been reported that the retention of Hill-activity in cell-free extracts is proportional to the degree of association between the biliproteins and chlorophyll. A parallelism between biliprotein release and loss of Hill activity suggests that analysis of the structural relationship, *in situ*, between phycocyanin and chlorophyll containing components might also help to clarify the structural relationship between the oxygen evolving processes and photophosphorylation.

The sub-microscopic morphology of *Anacystis nidulans*, when grown in continuous culture with agitation under 1% CO_2 in air, is very consistent. A number of entities are consistently observed in ultra-thin sections: the axial region, irregular in outline, contains fine filaments similar to the "nucleoid" of bacteria; the contiguous zone is rich in small particles of high electron density and contains a few relatively large masses of high electron density; the peripheral cytoplasm is incompletely partitioned by two or three parallel triple-layered lamellae. The relationship of these intracellular structures and the components of sub-cellular fractions will be discussed. — Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

9:30. SMILLIE, ROBERT M. and J. A. BERGERON, Brookhaven National Laboratory, Upton, L.I., New York. Succinic dehydrogenase of the photosynthetic sulfur bacteria.

The green photosynthetic sulfur bacterium *Chlorobium thiosulfatophilum*, strain L, and the purple photosynthetic sulfur bacterium *Chromatium*, strain D, both contain particle-bound succinic dehydrogenase (SD). A comparison has been made of the intracellular distribution and some properties of SD from these two organisms. Of particular interest is the possible association of the enzyme with chlorophyll containing particles derived from these bacteria. Cells were broken either by sonication or in a French pressure cell and cell-free particulate preparations were obtained by differential centrifugation. SD activity was measured by coupling with either ferricyanide or cytochrome c, the latter in the presence of catalytic amounts of N-methylphenazonium methosulfate (PMS). The *Chlorobium* enzyme, but not the *Chromatium* enzyme, reduced cytochrome c in the absence of added PMS, although an increase in activity invariably resulted upon the subsequent addition of PMS to the reaction mixture. SD activity from either bacterium was inhibited by malonate, fumarate, oxalacetate and pyrophosphate, but different effects were found with p-chloromecuribenzoate. The particulate enzyme from *Chromatium* has been solubilized and some of its properties determined.

Particulate SD from either *Chlorobium* or *Chromatium* is not associated with the chlorophyll containing particles. Thus in cell-free preparations from *Chlorobium* the SD is localized in fragments whose size range is different from that of the chlorophyll bearing particles. Preliminary experiments suggest that these two particle types originate from different structural components within the cell. Related studies on *Chromatium* will be presented.

Differences in the pathway of succinate oxidation by *Chlorobium* and *Chromatium* will be discussed briefly. — Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

9:45. PARK, RODERIC B. and NING G. PON, Univ. of California, Berkeley, Calif. Studies on lamellar structures isolated from spinach chloroplasts.

Chlorophyll containing lamellar structures were isolated from spinach chloroplasts and studied with respect to their physical, chemical and biochemical properties. Electron microscopic techniques for localization of chlorophyll within the lamellar structure and the relationship of holochrome to the lamellar subunit will be discussed.

10:00. Recess.

10:15. SCHIDLOVSKY, G., Research Institute for Advanced Study, Baltimore 12, Md. Chloroplast lamellae in higher plants and algae—a common structural motif.

The lamellar systems in mature chloroplasts of higher plants and algae are compared by electron microscopy after OsO_4 or $KMnO_4$ and $UO_2(NO_3)_2$ fixation, liquid dehydration or simple air-drying, acrylic or epoxy resins embedding and thin-sectioning.

In all cases examined (Spinach, Bean, Tomato and *Aspidistra* leaves, as well as *Euglena, Chlorella, Scenedesmus, Ochromonas, Prophyridia, Anacystis* and Tx-27 cells) the structural unit of the system is a *lamella* which may be conveniently described as a *closed and flattened sac*.

A process of *fusion* or close juxtaposition of random areas of the surfaces of two (and up to 70 or more) adjacent lamellae gives rise to grana (i.e. stacks of lamellae) in higher plants as well as in green and brown algae. Those portions of the lamellae that remain unfused (intergranor lamellae) may either extend unaltered and terminate freely in the matrix, or be continuous throughout two or more grana. In primary leaves of bean seedlings recovering in light from germination and growth in total darkness, stacks of fused lamellae (i.e. grana) appear only after 3-4 hours of illumination, although a constant increase in the number of isolated lamellae is evident throughout the earlier stages of illumination.

Although an identical system of fused flattened sacs is observed in green and brown algae (*Euglena, Chlorella, Scenedesmus* and *Ochromonas*) the number of sacs per stack rarely exceeds 10 while the relative area of fused lamellar surfaces is greatly increased (per chloroplast).

In red and blue-green algae, (*Porphyridia, Anacystis* and Tx-27) a fusion of lamellar surfaces is also observed. However, it is the inner surfaces of the flattened sacs that fuse with each other, resulting in an obliteration of the inner space of the latter.

It is the *fusion area* that appears therefore as a likely site for the location of active photosynthetic pigments.

The present concept of the granum being a separate morphological entity proper to higher plants appears untenable. It should rather be thought of as a differentiation of the lamellar system due to a light dependent mechanism occurring at or near lamellar surfaces throughout a wide variety of plants and lower photosynthetic organisms.

10:30. GIBBS, SARAH P., Harvard Univ., Cambridge, Mass. Chloroplast development in the chrysophyte, *Ochromonas danica*.

When dark-grown cells of *Ochromonas danica* are placed in the light, their chlorophyll content increases about 80-fold (from 4.4×10^{-11} to 3.6×10^{-9} mg. chlorophyll *a* per cell). Concomitantly with this increase in chlorophyll, the single small proplastid-like body of the dark-grown cells develops

into a large lamellate chloroplast. The course of chloroplast development has been studied with the electron microscope. During the first 12 hours in the light, as chlorophyll increases 6-fold, small vesicles appear in the stroma of the proplastids and fuse to form single or paired discs. After two days in the light, when the chlorophyll content per cell has increased 40-fold, the chloroplasts are almost full-sized and are now traversed by bands of three appressed discs. During the next three days as the chlorophyll content of the cells increases to almost maximum (75-fold), the membranes of the discs approach each other more closely and become much more regularly aligned.

Evidence will be presented which suggests that the nucleus plays a role in the development of the chloroplast and that the synthesis of the lamellar discs occurs in association with dense particles, which may be ribonucleoprotein particles.

10:45. MARGULIES, MAURICE M., Smithsonian Institution, Washington 25, D.C. The effect of chloramphenicol on chlorophyll formation and on development of photosynthetic activity.

Etiolated leaves of *Phaseolus vulgaris* var. Black Valentine were placed in solutions of chloramphenicol or in water and irradiated for 24 hours. An inhibition of chlorophyll formation occurred at a chloramphenicol concentration of 10 μgm/ml. The degree of inhibition increased as the concentration of antibiotic was raised, reaching 60-80% inhibition at 4000 μgm/ml. The green pigments accumulated in the presence of antibiotic were chlorophylls a and b and were present in the same ratio as in leaves treated with water instead of chloramphenicol. However, the effectiveness of chlorophyll in catalyzing photosynthesis decreased with increase in concentration of chloramphenicol. Decreased effectiveness of chlorophyll was detectable at an antibiotic concentration of 20 μgm/ml. Chlorophyll formed in the presence of 400-4000 μgm/ml of chloramphenicol was essentially ineffective in promoting photosynthesis. Incubation of leaves, already green, with 4000 μgm/ml of chloramphenicol had only a small effect on the ability of leaves to photosynthesize.

Leaves greened in the presence of chloramphenicol did not differ in their content of TPN dependent glyceraldehyde-3-phosphate dehydrogenase from water treated controls. Levels of carboxydismutase were somewhat lower in antibiotic treated leaves. However, Hill reaction activity of a green particle fraction from leaves greened in chloramphenicol solution were only a tenth that of the same fraction from control leaves.

These findings will be discussed in terms of photocontrol of the biochemical development of plastids.

11:00. GIBOR, A. and S. GRANICK, The Rockefeller Institute, New York City, N.Y. Evidence for the presence of plastids in streptomycin "bleached" *Euglena* and observations on spontaneous inherited changes in the plastids of *Euglena*.

Euglena gracilis strain Z (Hutner's strain) which was permanently "bleached" by streptomycin treatment was grown continuously in the light in a rich organic medium. The streptomycin treatment has been considered to cause the permanent disappearance of plastids from this *Euglena* strain. We have found by phase and fluorescence microscopy that these cells contain structures resembling plastids. By cracking the cells open, phase microscopy reveals these structures as small (about 2 microns) vesicles containing one or several dense granules. After incubation of intact cells in the presence of delta-amino-levulinic acid about 10 red-fluorescing bodies, 1-2 microns in diameter could be seen in the fluorescence microscope indicating the presence of porphyrins. The "bleached" cells were faintly yellowish presumably due to carotenoids not only localized in eye-spots, but also in these bodies. On the basis of their morphology and biochemical properties we conclude that these bodies are plastids. Streptomycin thus causes the loss of ability of *Euglena* plastids to differentiate to chloroplasts rather than affects the reproduction of these organelles.

Quantitative studies have been made of the highly "mutating" green *Euglena gracilis* Z confirming qualitative studies of W. J. Robbins *et al.* (1953). These "mutants" were distinguished by colony size and pigmentation. Non-green clones upon repeated subculturing never regreened. Some yellow-brown clones when grown in light accumulated carotenoids in plastids as well as in eye-spots; the plastids also contained porphyrins as revealed by their red-fluorescence. Other yellowish clones contained carotenoids in plastids but no porphyrins. Pale white colonies had much less pigment in their plastids but the plastids could still be identified by phase microscopy. These variants may be arranged in a series showing a graded degree of ability of plastids to differentiate towards chloroplasts. These investigations were supported in part by an N. S. F. Research Grant GI-4418.

11:15. ROSEN, W. G. and K. A. SIEGESMUND, Marquette Univ., Milwaukee, Wis. Effects of streptomycin bleaching on the fine structure of *Euglena gracilis*.

Exposure of dark-grown *E. gracilis* cultures to 500 mg/l of streptomycin (SM) in darkness for three days renders the cells permanently devoid of chlorophyll and chloroplasts on subsequent growth in light on SM-free medium. We have recently found, however, that if the cells are transferred to SM-free medium five days before transfer to light they green normally, indicating that proplastids are unaffected by SM.

Normal and SM-bleached cells, grown in light or in darkness, have been examined in the electron microscope. SM-bleached cells, grown in light, contain numerous bodies composed of concentric lamellae (CL bodies). These differ from chloroplasts in that their lamellae lack electron-dense dots and are not coalesced, their limiting membrane is single, and they lack pyrenoids. CL bodies are also occasionally seen in normal, light-grown cells. They are absent in dark-grown normal and dark-grown SM-bleached cells. The CL bodies resemble those found by Klein (*J. Biophys. Biochem. Cytol.* 8:529, 1960) in etiolated *Zea* leaves in which greening was blocked by low temperature or by dinitrophenol. It is suggested that CL bodies in SM-bleached cells represent a derangement of chloroplast synthesis caused by SM blockage of chlorophyll synthesis.

CL bodies in normal green cells may represent a separate class of structures. They are most frequent in cells from older cultures, have a somewhat different organization, and appear to arise from mitochondria.

Electron micrographs of the fine structure changes caused by SM will be shown. Supported by NIH grant RG6596.

11:30. OWENS, L. D., U.S. Soils Lab., Beltsville, Md. Purification and partial characterization of the chlorosis-inducing substance in soybeans.

In 1956 the discovery of a bacterial-induced chlorosis in soybeans was reported. Johnson, H. W., *et al* (*Nature* 183, 308. 1959) later found that water extracts of nodules from chlorotic plants contained the chlorosis-inducing substance, and that it was stable to autoclaving and freezing.

The substance has recently been isolated and purified, largely by means of ion-exchange chromatography. Dowex-50 cation-exchange resin buffered at a neutral pH was used because the compound was found to be acid labile, and to a lesser degree base labile. Ammonium acetate was used for buffering the column and eluting off the compound. The biologically-active fractions were combined and desalted by sublimation of the buffer, leaving a white residue of the substance.

The chlorosis-inducing compound is soluble in water and methanol, slightly soluble in ethanol, and insoluble in acetone, ether, and other common organic solvents. It dialyzes through a membrane having 20 Å pores, indicating a molecular weight of below 1000. Its chromatographic properties on both ion-exchange resin and paper are similar to those of the basic amino acids lysine and histidine. Reaction with ninhydrin produces a greenish-brown color which emits a chartreuse fluorescence under U.V. light, unlike that reported for any amino acid. Pauly's test for imidazoles was negative. The infrared spectrum of the substance indicates the presence of carboxyl, and amino or amide groups.

Further characterization of the compound will be discussed.

11:45. BADENHUIZEN, NICOLAAS P. and ELLEN N. LAWSON, Univ. of Toronto, Ontario, Canada, and Univ. of the Witwatersrand, Johannesburg, South Africa. An interesting case of chlorosis in *Cynodon dactylon* (Pers.) from South Africa.

Both cultivated and wild forms are characterized in South Africa by the spontaneous production of chlorotic shoots which are sterile. The physiological and biochemical properties of the chlorotic leaves are very similar to those produced by amitol or streptomycin: increased water content, amino acids, and respiration; inhibition of chloroplast development and catalase activity; production of a quanidin derivative. An extract of chlorotic leaves depressed catalase activity to about 50%. Its active principle was found to be allantoin, which has a bleaching action when applied to the leaves. The production of allantoin is probably the result of a metabolic change, by which it is accumulated until a threshold is reached where it becomes active and irreversibly inhibits both protein- and porphyrin synthesis, giving the impression of a mutation. Since neither X-rays nor gamma rays had any influence, the process appears to run parallel with the bleaching of *Euglena* by streptomycin, and in that case outer conditions (lack of iron or nitrogen) should have an effect. Results of experiments to detect parasitic microorganisms or a virus, were negative. The phenomenon does not occur in Bermuda grass from the United States.

SESSION G4. Room 310, Memorial Center

GROWTH IV

A. Lang, *presiding*

8:30. CUMMING, BRUCE G., Plant Research Institute, Canada Dept. of Agriculture, Ottawa, Canada. Photoperiodic response in the genus *Chenopodium* as related to geographical distribution.*

Photoperiodic studies with selections of 35 species of *Chenopodium* suggest that, in the temperate regions of North America, the majority of the annual species are short-day plants (SDP). In SDP the longer daylengths of early and mid season will favour vegetative growth while the shorter daylengths of late season will stimulate flowering. *C. rubrum* from localities differing in latitude (and therefore daylengths) viz: (a) 62° 46′ N (Yukon), (b) 60° 52′ N (Yukon, (c) 60° 47′ N (Yukon, (d) 50° 10′ N (Saskatchewan, (e) 49° 58′ N (Manitoba), (f) 34° 20′ N (California), has been grown (i) in Petri dishes at temperatures of 15, 20, 25, 35°C and daylengths of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 hours, using fluorescent light of 250 ft-c and (ii) in soil under continuous light in the greenhouse at altering temperatures of ca. 15-23°C and subjected to one or a limited number of photoinductive 8 hr. SD. Ability to flower under extreme long days, stimulation of floral initiation by one or a few photoinductive cycles, rate of stem elongation, rate of leaf production, and, amount of lateral shoot formation, were in the general order (a) > (b) > (c) > (d) > (e) > (f). The size of leaves and amount of anthocyanin formation was greater in the more southerly than Yukon selections. The range of photoperiods resulting in floral initiation, and the shift in the optimum photoperiod for floral initiation according to temperature, were in the general order (a) > (b) > (c) > (d) > (e) > (f). At 20°C, for example, floral initiation occurred in (a) under the 6 hr-24 hr. daylengths within six weeks, but in (f) only under the 6 hr.-12 hr. daylengths within eight weeks. Furthermore, while the optimum photoperiod for rate of floral initiation in (a) was 14 hr. at 15°C but 24 hr. at 30°C, in (f) it was 8 hr. at 15°C and 10 hr. at 30°C.

8:45. KASPERBAUER, M. J. and W. E. LOOMIS, Iowa State Univ., Ames, Iowa. Interaction of vernalization and photoperiod in flowering of sweet clover.

Biennial varieties of *Melilotus alba* and *M. officinalis* behave as annuals on photoperiods of 18 to 20 hours. Exposure to 20 or 30 long (24 hr.) days is necessary for the subsequent production of macroscopic flowers, but flowering in photoperiodically inducted plants has continued for 50 or more days with 9-hour photoperiods. One unusual inbred strain of *M. alba* appears to be critically sensitive to ultra violet radiation, and flowers only in the greenhouse. The rapid development of storage roots and crown buds which occurs in autumn is a response to photoperiod and independent of temperature.

Exposing plants to October and November temperatures in the field, or growing them in a cold greenhouse at 40-45°F. for four weeks caused flowering with photoperiods down to 13 hours; shorter photoperiods were not tested. A temperature

of 50°F. was not effective. Since biennial sweet clovers flower normally in the field with maximum photoperiods of 15 to 16 hours, it is clear that vernalization is required for flowering in the field, and that this requirement is the cause of the biennial growth habit.

9:00. MAUNEY, J. R. and L. L. PHILLIPS. U.S. Dept. of Agriculture, N.C. State College, Raleigh, N.C. The influence of photoperiod and night temperature on flowering of *Gossypium*.

A survey was made of the flowering reaction of more than thirty species and varieties of *Gossypium*, representing the wild and cultivated cottons of the world. The plants were subjected to four environmental regimes and the position of the first flowering branch and the first open blossom recorded. The environmental treatments were as follows: A) 14 hour daylength-85°F. night temperature; B) 14 hour day-60°F. night temperature; C) 10 hour day-85° night; D) 10 hour day-60° night. All plants received high temperature greenhouse conditions during the day.

The flowering reactions of the various species to these environments fell into several groups. 1) Those types which were day-neutral and only slightly influenced by temperature, 2) Those which initiated only on short days and were not appreciably influenced by temperature. 3) Those which initiated only on short days and required cool nights for development of the flower initials to blossom, 4) Those which were day neutral but required cool nights for development of the floral forms. 5) Those which required both short days and cool nights for initiation of the floral forms. 6) Those which required the long days and cool nights for initiation and development of the floral forms. 7) Those which required the cool nights for floral initiation and short days for development of the floral buds to blossom.

No type was found which flowered more readily in the high night temperature than in the low.

9:15. BORTHWICK, H. A. and H. M. CATHEY, U.S. Dept. of Agriculture, Beltsville, Md. Dependence of photoperiod control of flowering in chrysanthemum on dark reversion of phytochrome.

Flowering of chrysanthemums can be prevented by irradiating the plants with red light either continuously or intermittently for several hours near the middle of each long night. Such irradiation treatments convert phytochrome to the far-red-absorbing form, which is believed to be active in inhibiting flowering. Far-red-absorbing phytochrome is unstable and in darkness reverts to the red-absorbing form. The effectiveness of intermittent red irradiations lies in the fact that each successive irradiance returns the far-red-absorbing form of phytochrome to the flower-inhibiting level again.

The rate of dark reversion of phytochrome from the flower-inhibiting to the flower-inducing condition became the basis of so-called "cyclic" lighting of chrysanthemums. Plants were given light-dark cycles of various lengths in which a fixed total duration of light, regardless of cycle length, was given during a 4-hour period near the middle of each dark period. Cycle lengths of 1 to 30 minutes inhibited flowering when incandescent-filament light was the source of red. Cycle lengths of 1 hour and longer failed to inhibit flowering completely and were progressively less effective as the length increased.

The ratio of red to far-red radiation is considerably greater for fluorescent than for incandescent-filament light. Fluorescent light, therefore, converts a larger portion of phytochrome to the far-red-absorbing form. Reversion of this greater amount of far-red-absorbing phytochrome in darkness to the level non-inhibitory to flowering requires more time. For that reason longer light-dark cycles of fluorescent than of incandescent-filament light are effective. We tested other mixtures of red and far-red and found that as far-red increased relative to red the maximum length of effective cycle decreased.

9:30. ZABKA, GEORGE G. and J. EDELMAN, Univ. of Iowa, Iowa City, Iowa. Pre-illumination as a factor in the dark-fixation of CO_2 by *Kalanchoe blossfeldiana* var. Feuer Blute.

Using detached leaves of *Kalanchoe Blossfeldiana* var. Feuer Blute, the effect of illumination on CO_2 uptake during the dark period by leaf disc tissue was found to be restricted to a single preceding light period. Within certain prescribed limits, the amount of dark-fixation per unit quantity of light was constant whether this included a high intensity for short periods or relatively lower intensities for longer periods. Consequently, the CO_2 uptake was not dependent on the intensity or the duration of the preceding light period alone, but upon the product of these two factors within certain ranges used. The experiments indicate that a precursor for dark-fixation of CO_2 is formed by the action of light in the preceding photoperiod, and that, unlike the variety Tom Thumb, dark-fixation in Feuer Blute is independent of photoperiodic treatments.

9:45. ZEEVAART, JAN A. D., Calif. Inst. Tech., Pasadena, Calif. The effect of 5-fluorouracil on floral initiation in Japanese morning glory, *Pharbitis nil*.

Young seedlings (in cotyledon stage, 4 to 5 days old) of Japanese morning glory, variety Violet, respond to one single 16-hr dark period (temperature ca. 25°C) by initiation of 6 to 7 flower buds per plant. The initiation of flower buds can be completely inhibited by application of 5-fluorouracil (5-FU, concentration 10^{-3}M) either to the cotyledons or to the plumule before or shortly after the inductive dark period. Application to the plumule is more effective than application to the cotyledons.

Labeled 5-FU is rapidly translocated from the cotyledons to the plumule, but not in the reverse direction. This indicates that 5-FU does not interfere with the processes in the cotyledon which lead to the production of the floral stimulus, but that it inhibits the processes of floral differentiation.

5-FU inhibits both RNA and DNA synthesis in the plumule. It is moreover incorporated into RNA.

The 5-FU inhibition can be reversed by thymidine and by 5-methyldeoxycytidine, provided the application is made to the plumule before 5-FU can exert its inhibitory effect. The specific DNA inhibitor 5-fluorodeoxyuridine (5-FDU) has the same flower-inhibiting effect as 5-FU, except that it is effective already in a much lower concentration ($5x6^{-6}$M). These results suggest that the genes for floral initiation can be activated by the floral stimulus only while the DNA is multiplying.

10:00. Recess.

10:15. ROSS, CLEON W., Colorado State Univ., Fort Collins, Colo. Ribonucleic acid composition of vegetative and flowering *Xanthium* stem tips.

Previous inhibitor studies from this laboratory have indicated that nucleic acid metabolism is involved in the flower inductive process in *Xanthium*. The results suggested a modified RNA might be formed upon floral initiation and development.

The nucleotide composition of RNA was determined in terminal macroscopically visible flowers and compared with that of tips from vegetative plants. A modified Schmidt and Thannhauser procedure was used. The nucleotides were separated by paper electrophoresis, eluted, and measured spectrophotometrically. Very few studies have been made of RNA from higher plants, but the composition closely resembles that reported for tobacco leaf RNA (Reddi, K. K., *Biochem. et. Biophys. Acta*, 23:208, 1957). No significant differences were obtained between vegetative and flowering plants. RNA of the former contained 22.8 per cent adenylate, 23.9 per cent cytidylate, 30.8 per cent guanylate, and 22.5 per cent uridylate. Standard deviations were about 4 per cent. The preparations obtained do not follow Chargaff's rule. The ratio 6-amino/6-keto compounds averaged about 0.9 and was consistently less than 1.0. Two unknown UV absorbing spots were detected in all extracts. Some of their properties will be reported.

It is concluded that if differences in composition exist they are very small. It is possible that other differences are present or that only a fraction of the total RNA present is altered upon induction.

10:30. HARRISON, LEONA S. and FRANK B. SALISBURY, Colorado State Univ., Fort Collins, Colo. Studies on the mechanism of cobaltous inhibition in flowering of *Xanthium*.

Cobaltous ion inhibits the timing mechanisms in *Xanthium pennsylvanicum* Wall. (the critical night is extended, and the sensitivity to a light interruption of the dark period increases more slowly in plants treated with cobaltous ion), and its effects are reversed by simultaneous application of cysteine.

Present work was designed to investigate the nature of the cysteine reversal and the mechanism of the cobaltous ion effect on timing. Cysteine is known to complex with cobaltous ion in solution, and it was shown that the damage caused by cobaltous ion appears more slowly in plants treated simultaneously with cysteine (about a two-day lag in appearance of damage). Yet other amino acids known to form stable complexes with cobaltous ion were quite ineffective, although aspartic acid seemed to provide some reversal. Thus, complexing alone is probably not a sufficient explanation for the reversal of cobaltous ion effects. Since cysteine is a reducing agent, ascorbic acid was used in an attempt to reverse the cobaltous effect, and rather complete reversal was obtained. Thus the cobaltous effect may be related to the oxidation-reduction status of the leaf during timing.

Other substances, including colchicine and urethane (ethyl carbamate), have been reported to slow timing phenomena in leaf movements of bean, etc. Although colchicine caused vegetative responses, neither compound had any effect on flowering of *Xanthium*. Selenite catalyzes the oxidation of sulfhydryl groups and is a powerful inhibitor of flowering, although time of application experiments indicate that its action is not similar to that of colbaltous ion.

10:45. HILLMAN, WILLIAM S., Yale Univ., New Haven, Conn. Heavy metals and the photoperiodic control of flowering in *Lemna*.

Chelating agents confer photoperiodic sensitivity on two species of *Lemna* in a Hoagland-type medium, causing strict short-day dependence of flowering in an otherwise daylength-indifferent strain of *L. perpusilla* and long-day flowering in an otherwise non-flowering strain of *L. gibba* (Hillman, *Amer. Jour. Bot.* 48(5), 1961). In practically purified medium, these plants appear photoperiodic even without the chelating agents: *L. perpusilla* flowers much less rapidly in long than in short days, and *L. gibba* flowers rapidly in long days. Under these conditions, about 10^{-6} *M* cupric or mercuric ion *promotes* flowering in *L. perpusilla* in long days but has no effect in short, and inhibits *L. gibba* flowering in long days. Effects on vegetative growth are minimal. Thus these ions may be relatively specific inhibitors of photoperiodism, and the action of chelating agents observed earlier probably represents prevention of their effects as contaminants in the medium. A number of other heavy metals, including cadmium, have not shown similar effects.

Rigorous purification of the medium has not yet been undertaken, and variance in many experiments is undesirably high. In addition, effects of other components of the mineral medium on the apparent copper (or mercury)-photoperiod interaction require further study. However, this system under better control may well provide new tools for the analysis of the photoperiodic control of flowering, at least in *Lemna*, and hopefully in other plants as well.

11:00. ROBERTS, R. H. and B. ESTHER STRUCKMEYER, Univ. of Wisconsin, Madison 6, Wis. Induction of flowering by natural lipid hormones.

Oil extracts from plants in blossoming stages from induction to flowering have yielded three lipid hormones which induce and accelerate blossoming. The hormones are applied by spraying with an aqueous suspension. More than 70 species or varieties of plants representing more than 20 families have been induced. The anthogens (Greek, blossom generating) are interchangeable in effects, as extracts from both long- and short-day plants induce both types. Plants should be treated when young to avoid natural induction prior to treatment. The accelerated growth which accompanies natural induction is also produced by hormonal induction. Gibrel can be used on some plants to further hasten flowering; when used with anthogens good flowers are secured in contrast to the usual poorly developed ones from Gibrel alone. It develops that photoperiodism is not just the hormonal stimulus to flowering. It is particularly the synthesis of precursor metabolites upon which the anthogens can react.

11:15. ROBERTS, R. H., Univ. of Wisconsin, Madison 6, Wis. Photoperiodism, in view of recent lipid hormone induction studies.

The hormone which induces blossoming has been isolated from flowering plants by oil extraction. It has induced over 70 species or varieties of plants representing more than 20 families. This does not, however, solve the problem of photoperiodism. Two of several reasons why photoperiod effects are more than hormonal stimulation follow: 1. The anthogens, (Greek, blossom generating) extracted from both long- and short-day plants are interchangeable in their induction of the other. 2. Also, they do not readily induce blossoming unless the treated plants have been grown for a time in the typical photoperiod. It appears that photoperiod is basically the synthesis of metabolites from which the anthogens can initiate blossoming. What would be the precursors to flowering that react with lipid hormones?

11:30. STUART, NEIL W., Agricultural Research Service, Beltsville, Md. Initiation and development of flower buds in *Rhododendron* following application of growth retardants

Application of the growth retardants phosfon (tributyl-2,4-dichlorobenzylphosphonium chloride and CCC [(2-chloroethyl) trimethylammonium chloride] as soil drenches caused suppression of vegetative growth and prompt initiation of flower buds in azalea, *Rhododendron* spp. These responses did not depend on minimum age or size of plant, specific photoperiod or temperature and occurred under environmental conditions which often prevented or limited flower bud initiation in untreated plants. Normal flowering followed exposure of the treated plants to dormancy-breaking cool storage. Vegetative shoots developed after flowering at rates inversely proportional to the concentration of the applied retardant.

11:45. SCOTT, R. A., Jr., Boll Weevil Research Laboratory, Starkville, Miss. Reversal of the nondehiscence of anthers by application of choline chloride or urea on cotton leaves.

At a minimum concentration of 100 micrograms per plant, choline chloride or urea applied to the foliage of cotton plants is able to reverse a naturally occurring nondehiscent-anther condition. The expression of this disorder appears to relate to the nitrite-ion concentration in the second and third foot of soil. The condition is also correlated with water deficiencies which are often associated with poor penetration of water into the soil and poor soil aeration. Timing of the applications and seed cotton yields are presented.

12:00. MOORE, J. N. and L. F. HOUGH, Rutgers—The State University, New Brunswick, N. J. Influence of gamma irradiation and short-day inductive treatments on endogenous auxin levels and vegetative and reproductive growth in the strawberry.*

Sparkle strawberry plants were given 0r/day, 100r/day, 500r/day, and 1000r/day of gamma irradiation for 42 days and then subjected to various numbers of short-day inductive cycles. Following short-day treatments, methanol extracts of the plant crowns were chromatographed and growth regulator content was determined by the *Avena* first internode assay method. Irradiation at 1000r/day resulted in an initial decrease in auxin levels, but recovery was complete 7 days after treatment. Auxin levels were less influenced by short photoperiod treatment in plants previously subjected to high irradiation dosages. Plants irradiated at 500r/day required a greater number of consecutive short-day cycles for floral induction to occur than did the control and 100r/day treatment. Plants given 1000r/day failed to flower regardless of the number of photocycles given. A suppression of vegetative growth was observed in all irradiated plants and could not be correlated with auxin content.

Index of Authors

Abbadi, S. xix
Aghion, J. iv
Albersheim, P. xxxix
Anderson, D. G. xlv
Anderson, I. C. xliv
Andreae, W. A. xl
Arnon, D. I. xi
Aronoff, S. xxvi
Arya, H. C. xxix
Ashton, F. M. xli
Austin, S. W. xxiv

Badenhuizen, N. P. li
Baker, J. E. ..... xix, xxxii
Bannister, T. T. ..... iii, iv
Barber, G. A. xxxvii
Barnes, R. L. xviii
Beevers, H. xxxi
Bell, W. D. xv
Benda, G. xxiii
Benson, A. A. xlvi
Benton, G. S. ix
Ben-Yehoshua, S. xxx
Berezniak, H. v
Bergeron, J. A. ..... xlviii, xlix
Bernstein, L. xxiii
Bertsch, W. F. iv
Biale, J. B. xxx
Bieleski, R. L. xxx
Black, C. C. x
Black, M. K. xxxix
Blumenthal-Goldschmidt, S. ..... vii, viii
Bonner, B. A. xliii
Bonner, J. xxi
Borthwick, H. A. lii
Bourke, M. xix
Brewbaker, J. L. xvi
Brown, J. C. xiv
Brown, O. D. R. xxi
Brown, T. E. iii
Brun, W. A. xxiii
Buckley, E. H. xxxviii
Bukovac, M. J. vii
Bula, R. J. xix
Burris, R. H. xxviii
Bushnell, W. R. xiii
Butler, W. L. ..... iv, xlii

Campbell, R. W. viii
Castle, J. E. xlvii
Cathey, H. M. ..... xxxviii, lii
Chen, J. xxvii
Cherry, J. H. vi
Ching, T. M. xlv
Clagett, C. O. ..... ix, xviii, xxxi
Clements, R. L. xx
Cobb, H. D. xxv
Coniglio, J. G. xlvi
Correll, D. L. v
Cox, E. A. xxi
Craft, C. C. xxxviii
Crane, F. L. x
Culbreth, W. xxxiii
Cumming, B. G. li
Curtis, R. W. xiii

Das, M. L. x
Dawson, W. R. xvii

Index of Authors

Davis, J. T. xlvi
Decker, J. P. xvii
Downs, R. J. xlii
Dugger, W. M. ..... xxvi, xliv
Duysen, M. E. xiii
Dycus, A. M. xii

Edelman, J. lii
Ehlig, C. F. xxiv
Eilberg, R. xvii
Epstein, E. xxxiv
Etherton, B. ..... xxxv, xxxvi
Eyster, C. iii

Fewson, C. A. ix
Finn, J. C. xxi
Firer, E. M. xlii
Flemion, Florence xxvii
Fredericks, W. W. iii
Freebairn, H. T. xx
Foster, R. J. xxxv
Foy, C. L. xl
Fu, Ming Hwa xli
Furuya, M. xlvii

Gagliardi, N. C. ..... vi, vii
Galloway, R. A. xxxiii
Galston, A. W. xlvii
Garver, J. C. xlvi
Gettens, Rebecca xlii
Gibbs, M. ..... ix, x
Gibbs, Sarah P. xlix
Gibor, A. l
Good, N. E. xi
Goos, R. D. xxi
Gordon, S. A. xlvi
Granick, S. ..... xlviii, l
Grant, B. R. xxxi
Grant, D. R. xviii
Green, P. B. xxvii
Greig, Ethyl E. xxxvi
Greulach, V. A. xiv
Griffith, M. M. xliii
Gruen, H. E. xxii
Guerrero, D. xxii
Gupta, P. K. viii

Haber, A. H. xl
Hageman, R. H. vi
Hagen, G. L. xxviii
Hanson, J. B. xxxii
Harley, J. L. xxxi
Harrison, Leona S. liii
Hauschild, A. H. W. xxvi
Havir, E. A. x
Hayashi, F. vii
Heck, W. W. xxiii
Hendricks, S. B. xlii
Hepler, P. K. xxviii
Herndon, W. R. xvi
Hess, C. E. xxi
Hiatt, A. J. xxxvii
Hiesey, W. M. xxvi
Higinbotham N. xxxv
Hildebrandt, A. C. ..... xxii, xxviii, xxix
Hillman, W. S. liii
Hoch, G. xii
Hodges, T. K. xvi
Honda, S. xlviii
Hongladarom, T. xlviii
Horton, A. A. xi
Hotta, Y. v
Hottinger, H. H. xxix
Hough, L. F. liv
Hudson, W. xix

Ikuma, H. xlii
Inselberg, E. xv

Jacobson, A. B. xl
Jagendorf, A. T. ..... iii, x, xi
Janes, B. E. xxiv
Joven, C. B. xv

Kahn, J. S. x
Kapoor, M. v
Kasperbauer, M. J. li
Kato, J. xiii
Katz, J. M. xli
Kaufman, P. B. xli
Kearney, P. C. xxvi
Kelly, S. xxxvii
Ketellapper, H. J. xxi
Key, J. L. xii
Kidder, G. W. xxxii
Kim, W. K. xiv
Kindel, P. K. ix
King, J. v
Klein, A. O. v
Klein, W. H. xlii
Kohl, H. C. xliv
Kok, B. xii
Krall, A. R. x
Kramer, P. J. xvii
Krauss, R. W. ..... xxxiii, xliii
Kress, L. F. xxviii
Krogmann, D. W. xi
Krotkov, G. ..... xxvi, xxxi, xxxiii
Krulick, S. xvii
Kumar, S. xxii
Kuraishi, S. xlvi
Kwack, B. H. xvi

Lam, S. L. xl
Lang, A. ..... xii, xxv
Laties, G. G. xxx
Lawrence, J. M. xviii
Lawson, Ellen N. li
LeFevre, C. W. ix
Leggett, J. E. xxxv
Leland, H. V. xx
Leopold, A. C. ..... xiv, xl
Linder, P. J. xxxiv
Lippincott, J. A. iv
Lister, G. xxvi
Lister, G. R. xxxiii
Lockhart, J. A. xxxviii
Loercher, L. xliii
Loewus, F. A. xxxvii
Loomis, W. E. ..... xxii, li
Love, B. B. iv
Low, P. F. xix
Lyon, C. J. xx
Lyons, J. M. xxx

Machlis, L. xxvii
Mans, R. J. xvii
Marcus, A. xxxvii
Margulies, M. M. l
Marumo, S. xiii
Mascarenhas, J. xxvii
Mauney, J. R. lii
McComb, E. A. xxxvi
McGlasson, W. B. ..... xxix, xxx
Michel, B. E. ix
Millar, Elnora xix
Miller, E. V. xlv
Milner, H. W. xxvi
Minshall, W. H. xvi
Mish, L. B. xxxiii
Mitchell, J. W. xxxiv
Mitchell, R. L. xliv
Moore, J. N. liv
Morris, C. J. xviii
Mortimer, D. C. xxxiv
Mumford, F. E. xlvii
Murashige, T. xxviii
Myers, J. xxv

Nance, J. F. xxxix
Nandi, D. L. xlvi
Naylor, A. W. ..... xviii, xxv
Nelson, C. D. ..... xxvi, xxxi, xxxiii
Nepokroeff, C. M. vii
Newcomb, E. H. xxviii
Ng, M. Y. L. xlvi

Oaks, Ann xviii
Oberbacher, M. F. xxxi
O'Kelley, J. C. xvi
Olah, S. E. xlv
Owens, L. D. li

Pallas, J. E. xxxvi
Palmer, J. K. xxxviii
Park, R. B. xlix

Index of Authors

Perry, R. L. xxiv
Phillips, L. L. lii
Phinney, B. O. xiii
Pires, E. G. xxiii
Plaisted, P. H. v
Pon, Ning G. xlix
Porter, J. W. xlv
Porter, W. L. xxxix
Posner, H. B. xxii
Powell, L. E. vii
Powell, R. D. xliii
Pratt, H. K. ..... xxix, xxx
Price, C. A. ..... xix, xxxvii
Punnett, T. xxxvi
Purves, W. K. xiii

Raney, F. ..... xvi, xxiv
Raney, F. C. xxiv
Rappaport, L. ..... vii, viii
Ray, P. M. xxvii
Ree, B. J. iv
Reed, D. J. xxxii
Reggio, R. v
Reinhard, E. xii
Rendig, V. V. xxxvi
Riker, A. J. ..... xxii, xxviii, xxix
Ritzert, R. W. vii
Roberts, R. H. liii
Robertson, R. M. xxx
Robinson, Melba B. xxxiv
Roderick, T. H. xxix
Rogers, B. J. xl
Rosen, W. G. ..... xxviii, l
Ross, C. W. liii
Rowan, K. S. xxix
Rubinstein, B. xiv
Russell, M. B. ix
Russo, M. J. xii

Sahai, B. I. viii
Salisbury, F. B. liii
Sansing, N. G. xxxiii
Santilli, V. ..... vi, vii
Schidlovsky, G. xlix
Schopmeyer, C. S. xxxiii
Schrank, A. R. ..... xx, xli
Scott, R. A. liv
Sell, H. M. vii
Shafer, J. xix
Shafer, R. B. iv
Shannon, L. M. xxxvii
Shaw, M. viii
Shiroya, M. xxxiii
Shiroya, T. xxvi
Shropshire, W. xli
Siegelman, H. W. xlii
Siegesmund, K. A. l
Sievert, R. C. xxviii
Sisler, E. C. xlii
Skok, J. xv
Smillie, R. M. ..... xlviii, xlix
Smith, D. H. xlvii
Sorokin, C. ..... xxv, xliii
Spurr, H. W. xxix
Stanley, R. G. xvi
Stern, Babette, ... iii
Stern, H. v
Stiller, Mary xi
Stolzy, L. H. xxxv
Stotzky, G. ..... xxi, xxxiii
Stowe, B. B. xlvii
Strong, F. M. xlvi
Struckmeyer, B. Esther liii
Stuart, N. W. liv
Surrey, K. xlvii

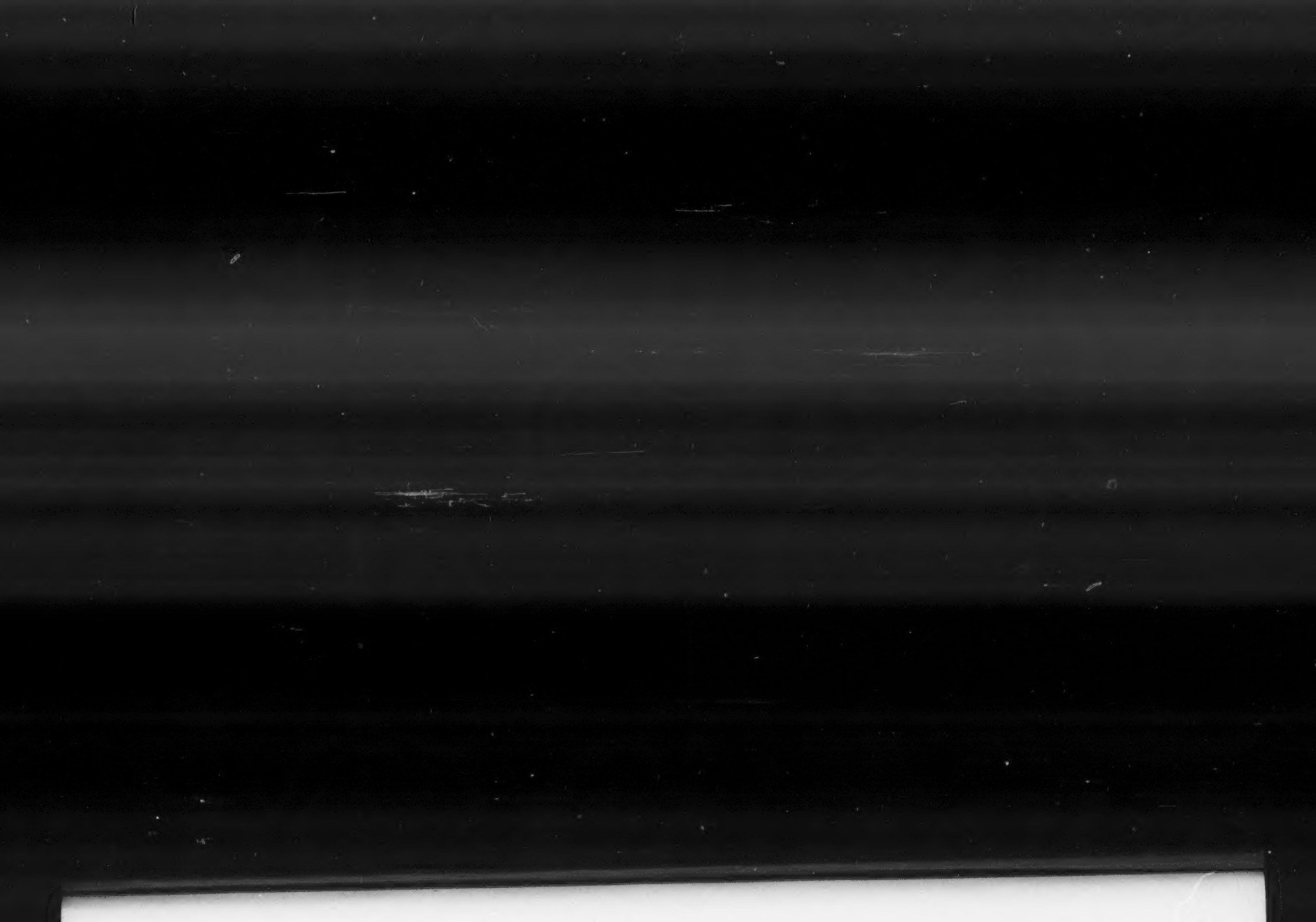
Tanada, T. ..... xxxiv, xxxv
Taylor, O. C. ..... xxvi, xliv
Thimann, K. V. ..... xxxix, xlii, xlvi
Thomas, M. D. xxvi
Thompson, C. R. xxvi
Thompson, J. F. ..... xviii, xix
Tiffin, L. O. xiv
Todd, G. W. xxiv
Tolbert, N. E. xxvi
de Torok, D. xxix
Tregunna, E. B. xxxi
Turner, J. F. x
Turrell, F. M. xxiv

Vaadia, Y. xvi
Van Noort, D. xv
Van Noort, G. xix
Van Norman, R. W. iv
Van Ysselstein, M. W. H. xl
Vargas, M. xxxi
Vines, H. M. xxxi
Vishniac, W. v
Vrooman, M. J. iii

Waggoner, P. E. ix
Waisel, Y. xxxv
Walker, R. B. xv
Wallace, A. ..... xv, xix
Wallihan, E. F. xv
Wang, D. ..... v, xvii
Waygood, E. R. ..... v, xlvi
Wedding, R. T. xxxix
Weinstein, L. H. xlv
Went, F. W. xvii
Whatley, F. R. xi
Whitehead, C. W. xxxiii
Wickliff, J. L. xxvi
Wiegand, O. F. xli
Wildman, S. G. ..... xix, xlviii
Wilson, C. M. vi
Wittwer, S. H. xlv
Wolf, F. T. ..... xxxix, xlvi
Woolley, J. T. xxv

Yagi, T. xlvi
Yoo, B. Y. xxiii
Young, R. E. xxx
Yu, P. K. viii

Zabka, G. G. lii
Zacharius, R. M. xvii
Zaroogian, G. E. xiii
Zeevaart, J. A. D. lii
Zill, L. P. xxv



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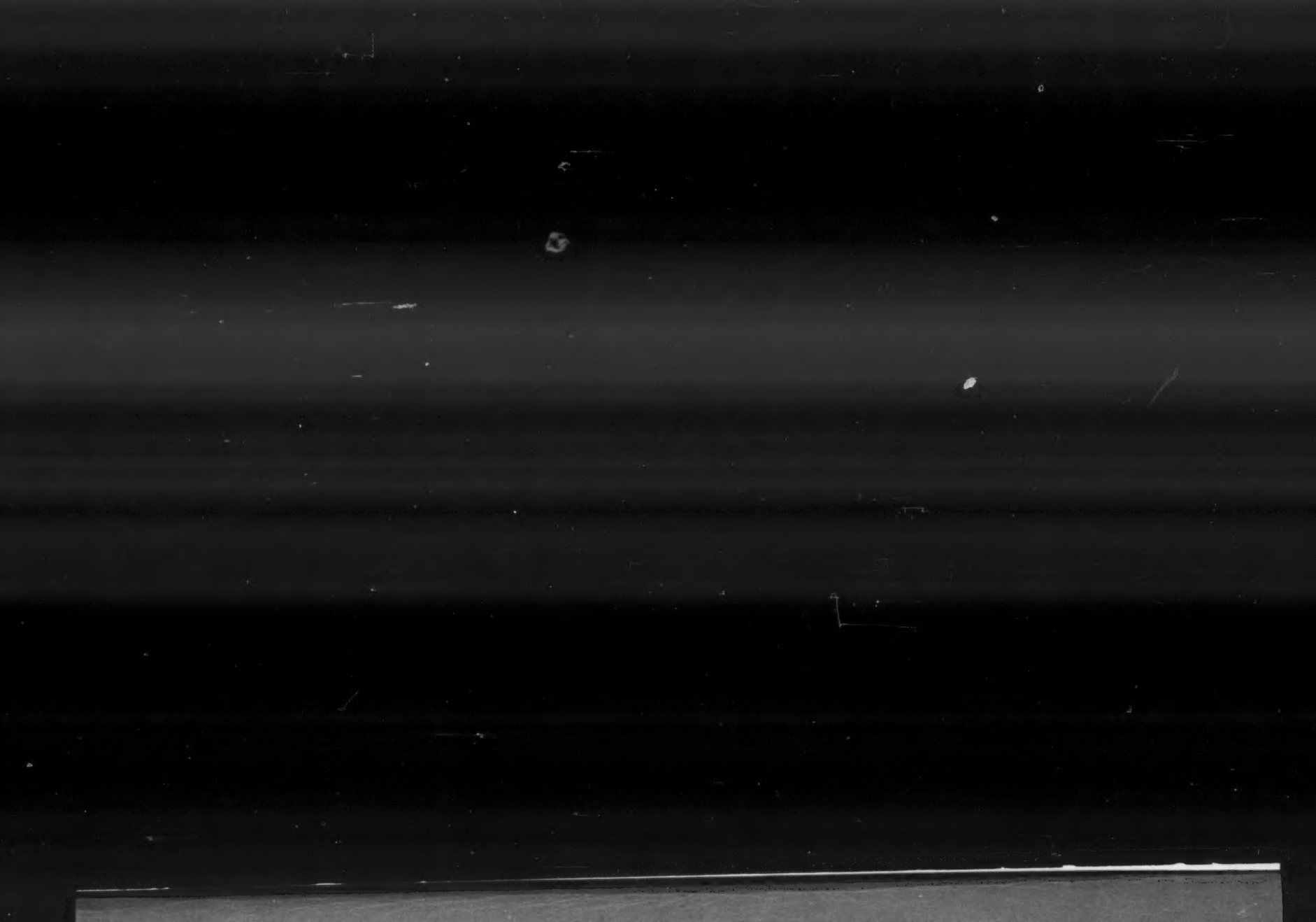
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